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14. ABSTRACT This study focuses on the enzyme, CYP24 which hydroxylates vitamin D acting to catalyze the first step in the breakdown of Vitamin D, effectively limiting this growth inhibitory signaling pathway. We are testing the hypothesis that through the inhibition of CYP24 using an siRNA approach we can convert prostate cancer cells that are resistant to the antiproliferative actions of Vitamin D to cells that are growth inhibited at low concentrations of Vitamin D. Inhibition of 1,25(OH)2D3 CYP24 mediated metabolism to potentiate Vitamin D actions in prostate cancer shows great potential for both a chemopreventative approach and the treatment of advanced hormone refractory cancer in patients. We have tested CYP24 siRNA constructs, ketoconazole and silencer control siRNA on three cell lines (LNCaP, PC3 and DU145) and evaluated CYP24 protein expression, mRNA expression, and growth inhibition. We are in the process of developing the stable transfected cell lines and optimal approach to enhance Vitamin D action in resistant cells.					
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FINAL PROGRESS REPORT

Specific Aim #1 *To enhance Vitamin D inhibition of prostate cancer growth through inhibition of CYP24.*

The summary below describes our progress towards meeting all aims on our funded proposal.

1. CYP24A1 is Over-expressed In Human Prostate Cancer: It was important to first assess the mRNA levels of endogenous CYP24A1 in adenocarcinomas of the human prostate. cDNAs obtained from pathologist-verified human prostate biopsies representing different TNM (Tumor, Node and Metastasis) stages of prostate cancer, as well as, benign prostatic hyperplasia (BPH) were analyzed by quantitative PCR. Despite the variability observed between individuals, the benign tissues expressed low levels of CYP24A1 while the majority of the analyzed adenocarcinomas (73%) had CYP24A1 highly expressed (Figure 1A). When the relative levels of mRNA were examined as mean values by TNM stage, a marked elevation of CYP24A1 was observed with increasing pathological grades of prostate cancer, with some heterogeneity at T3 (Figure 1A). Consistently, CYP24A1 protein was over expressed in adenocarcinomas of prostate compared to normal human prostate (Figure 1B). While normal cells demonstrated faint cellular staining for the catabolic enzyme, immunodetection of CYP24A1 revealed intense cytoplasmic staining in epithelial cells of both low and high Gleason grade adenocarcinomas (Figure 1B).

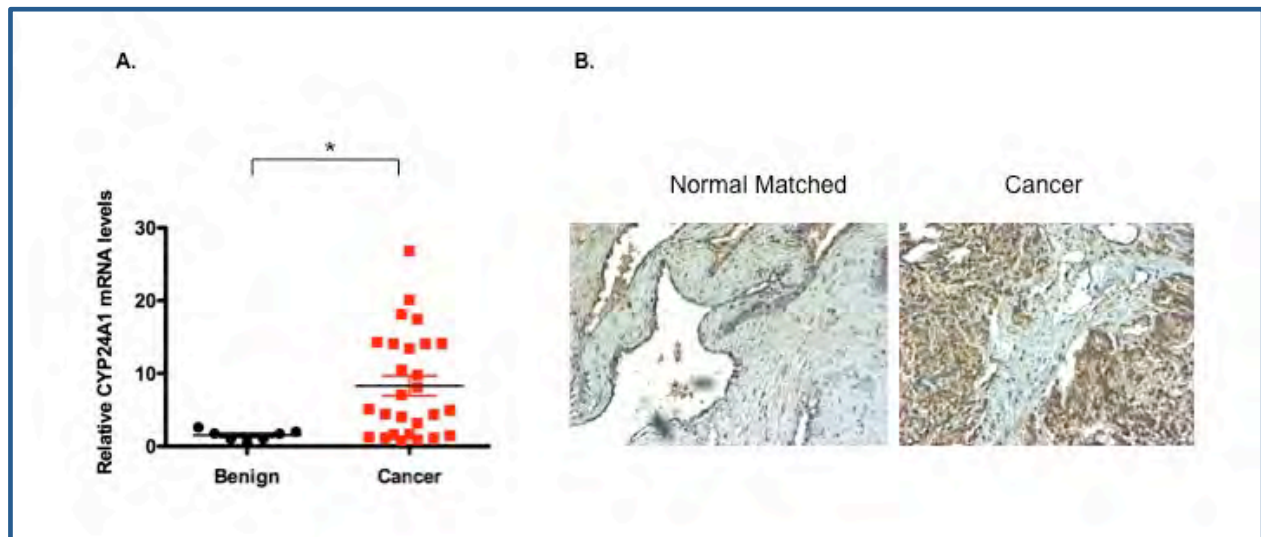


Figure 1: Over expression of CYP24A1 in men with prostate cancer. A. CYP24A1 gene expression levels across the stages of human prostate cancer progression. TaqMan QPCR was performed using specific CYP24A1 primers. β actin served as an internal control. Patients' cDNA were prepared from well-documented cancer biopsy samples, normalized and assembled into ready-to-use gene expression panels. Clinicopathological information for each patient was provided by the supplier (Origene, MD, USA). Data were analyzed using the comparative CT method with the values normalized to β actin levels. Figure in inset is a scatter plot presenting qPCR results as averages of relative CYP24A1 mRNA levels in individuals presenting identical TNM stage. **B. Marked increase of cytoplasmic CYP24A1 immunostaining (brown) in representative human prostate adenocarcinomas compared to normal human prostate.**

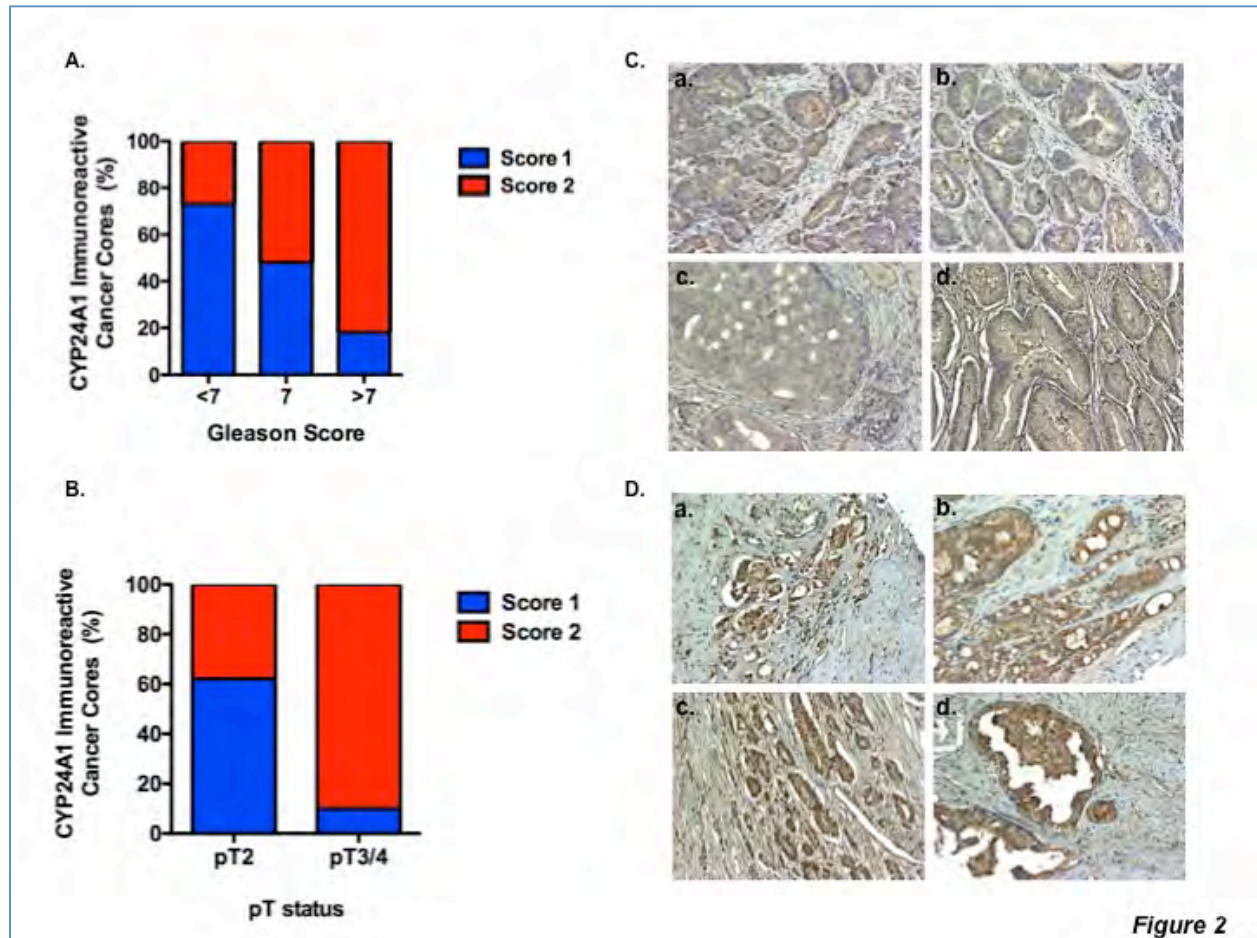
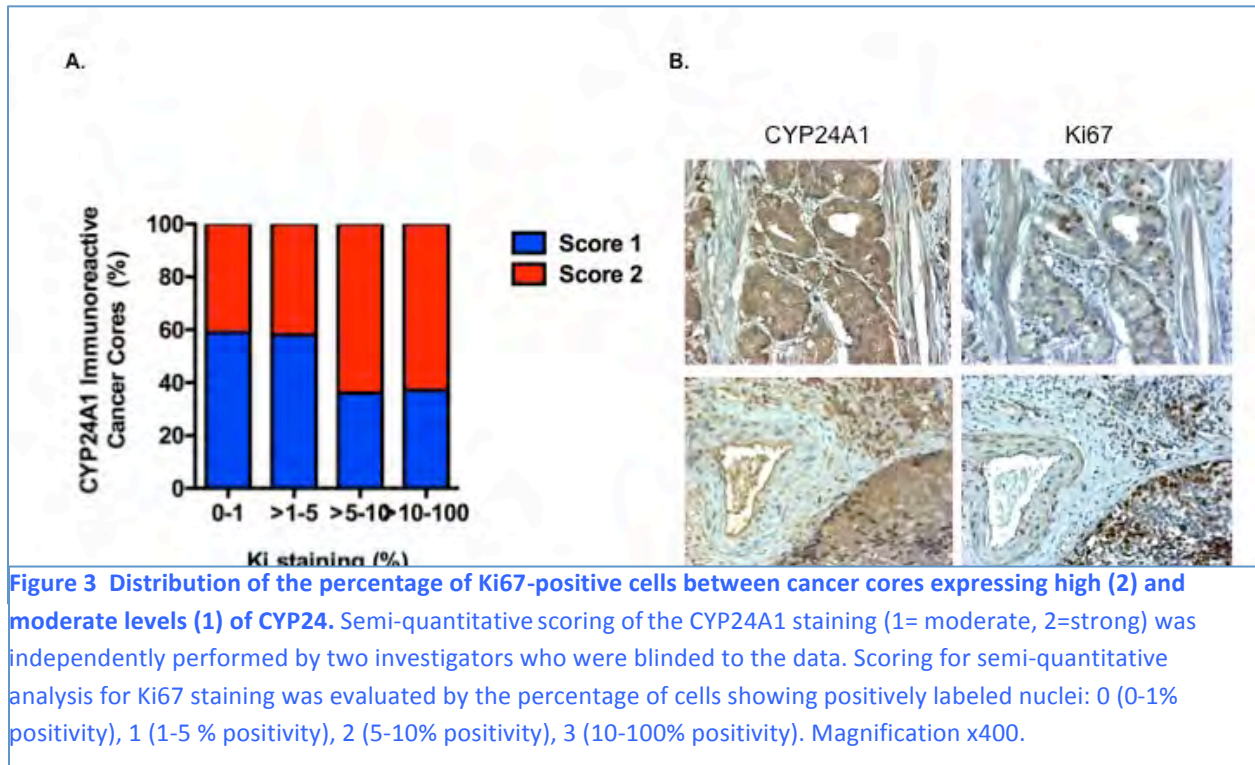


Figure 2

Figure 2: Correlation between high protein levels of CYP24A1 and high tumor grade and late stage of human prostate cancer. A-B. Distribution of the relative expression levels of CYP24A1 and Gleason score (A) and tumor stage (B) categories. Semi-quantitative scoring of the quantity of CYP24A1 expression in 112 prostate cancer cores, derived from 56 patients with localized and metastatic prostate cancer disease, was performed (score 1= moderate; score 2=strong). Cases are shown as percentages of total immunoreactive cancer cores on the y-axis. C-D. Immunostaining of CYP24A1 in representative adenocarcinomas of different grades. **In C., low grade lesions of prostate cancer showing moderate intensity of the staining of the catabolic enzyme** (a. Gleason 2+2 and T2NxM0; b. Gleason 2+3 and T2NxM0; c. Gleason 2+3 and T2NxM0; d. Gleason 1+1 and T2bNxM0). **In D., intense staining in malignant prostate tissues presenting with high Gleason score and late stage tumor progression** (a. Gleason 9 and T3bN0M0; b. Gleason 7 and T3bN0M0; c. Gleason 7 and T4N0M0; d. Gleason 9 and T3bN0M0). Magnification x400.

Indeed, this is seen clearly in Figure 2 that shows the correlation of CYP24A1 with high tumor grade and late stage prostate cancer. We analyzed tissue samples from 112 prostate cancer cores, derived from 56 patients with localized and metastatic prostate cancer disease and Gleason scores ranging from 4 to 10. The percentage of immunoreactive cases with CYP24A1 was high (98% of cancer cores). However, staining intensity varied between patients, ranging from intense immunoreactivity of the entire cytoplasm to more discrete granular staining. Importantly, high protein levels of CYP24A1 positively correlated with high-grade cancer (Figure 2; $P < 0.0001$, Pearson's Chi-square), as well as with late stage tumor progression (Figure 2; $P < 0.0001$, Pearson's Chi-square). Increased CYP24A1 staining intensity was indeed, frequently observed in cancer cores with Gleason scores higher than 7 and in lesions presenting with 3 or 4 TNM stages (Fig 2A-D). Taken together, these data demonstrate for the first time, the



changes of expression of CYP24A1 in patients with prostate cancer and highlight the potential key role of CYP24A1 in prostate cancer.

2. Basal Levels of CYP24A1 mRNA Correlate With The Growth Response To Vitamin D In Prostate Cancer Cells: Next, to examine whether increased CYP24A1 expression reflects increased tumor proliferation, we determined its relationship to the expression of the proliferation marker Ki67. High expression of CYP24A1 positively correlated with parallel increased staining of Ki67 in the same human prostate cancer tissues (Figure 3; $P < 0.0002$, Pearson's Chi square). This observation supports the hypothesis that overexpression of CYP24A1 in prostate cancer cells may reduce the ability of 1,25-dihydroxyvitamin D₃ to inhibit proliferation.

As the steady-state levels of cellular vitamin D₃ in prostate tumor tissue of patients is difficult to measure, we analyzed the expression levels of endogenous CYP24A1 in three human prostate cancer cell lines (LnCAP, PC3 and DU145) and monitored their respective cell proliferation in presence of vitamin D₃. Consistent with the findings of previous reports on CYP24A1 enzymatic activity in these prostate cell lines, we found an inverse correlation between the constitutive cellular levels of CYP24A1 mRNA and the growth response to vitamin D₃. Indeed, LnCAP, with the lowest constitutive expression levels of CYP24A1 (Figure 4A), is most responsive to the anti-proliferative effect of calcitriol (Figure 4B). In contrast, PC3, which displays the highest basal levels of CYP24A1 (Figure 4A), is the most resistant to the inhibitory effect of calcitriol on cell growth (Figure 4D). Thus, the differential prostate cell response to calcitriol is due to their inherent constitutive gene expression levels of 1,25(OH)₂ vitamin D₃ catabolic enzyme, CYP24A1. These data support the role of vitamin D₃ as a paracrine hormone to inhibit the growth of the prostate and provide a rationale basis to the use of specific inhibitors of CYP24A1 to enhance vitamin D-based therapies for prostate cancer management.

2.a Optimal Transcript Design for Efficient CYP24A1 Inhibition: We first assessed by qRT-PCR, the efficacy of inhibition of CYP24A1 expression. siRNA oligonucleotides were

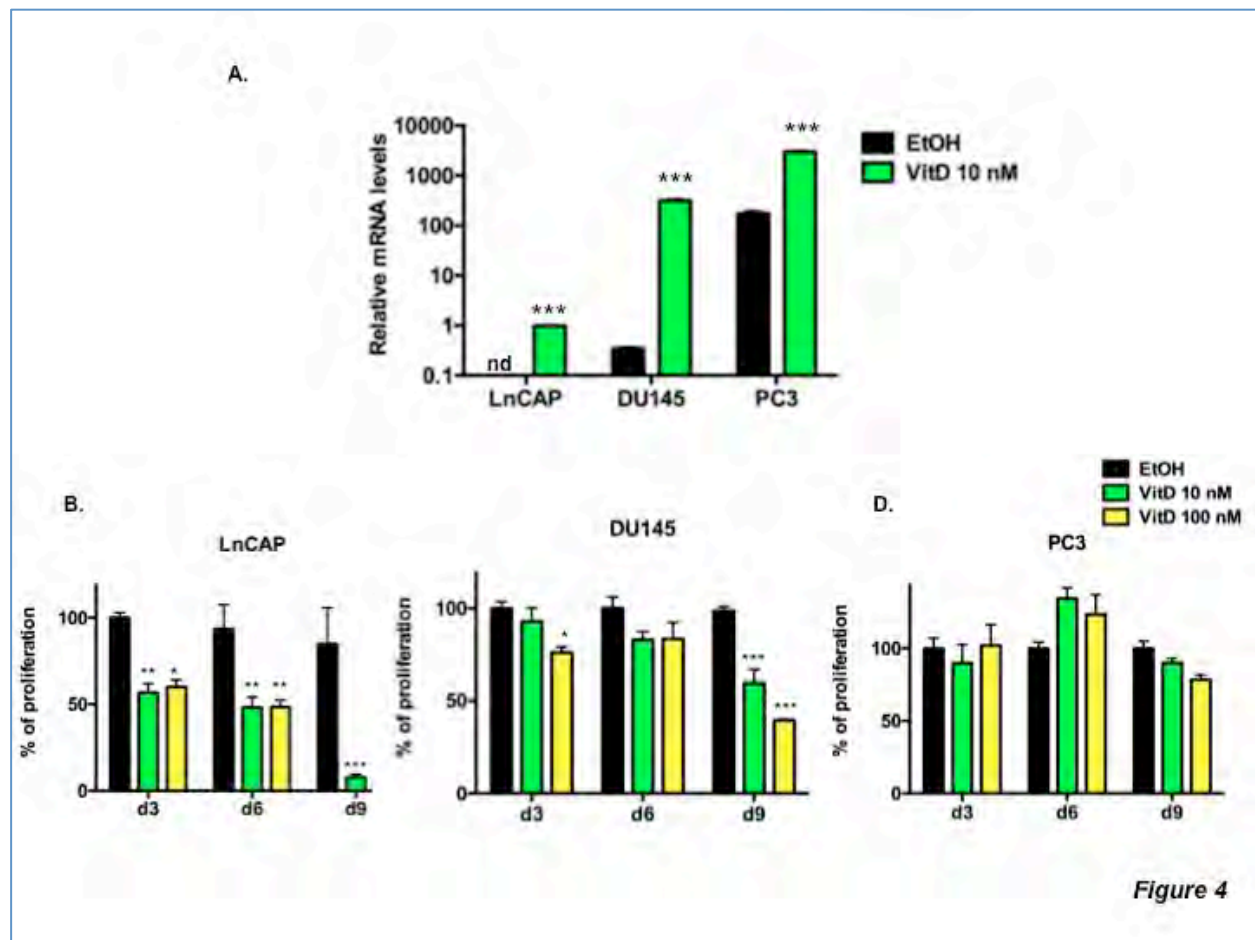


Figure 4: Inverse correlation between endogenous levels of CYP24A1 mRNA and growth abrogation action of vitamin D₃.

A. qRT-PCR analysis of endogenous CYP24A1 mRNA levels in presence of 10nM Vitamin D₃ or vehicle (ethanol; EtOH) in LnCAP, DU145 and PC3 prostate cancer lines. GAPDH served as the internal control. **B-D.** Cell proliferation assays performed in presence of 10 nM or 100 nM calcitriol or ethanol after 3, 6 and 9 days of treatment in LnCAP (B), DU145 (C) and PC3 (D) (* if $P < .05$; ** if $P < .01$; *** if $P < .001$, ANOVA).

synthesized by Dharmacon Research, Inc. (Lafayette, CO). Cells were transfected with CYP24A1siRNA oligos or scrambled siRNA. The optimal approach used CYP24A1 ON TARGETplus SMART pool at a concentration of 20-40 ng/ml

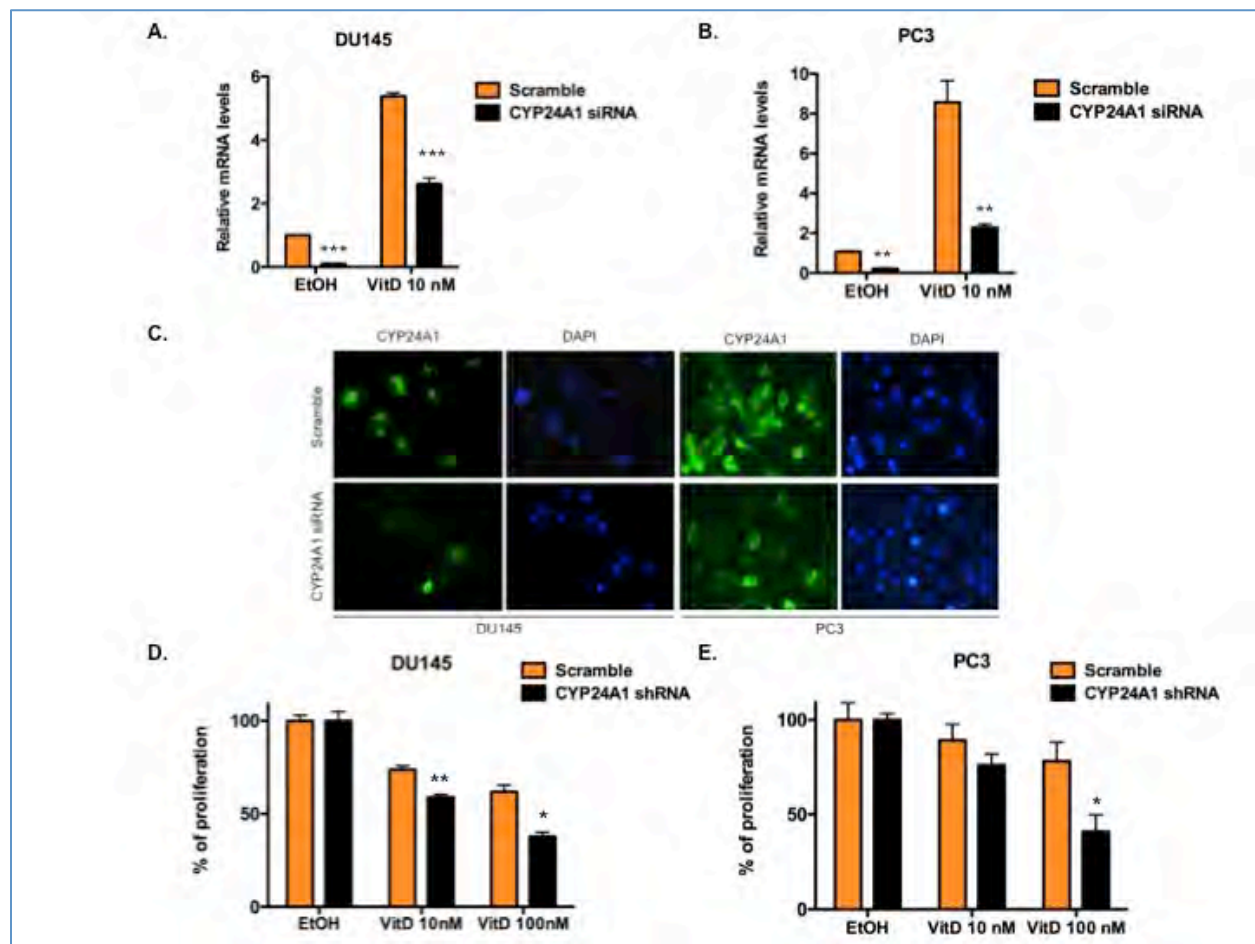


Figure 5: Effect of selective inhibition of CYP24A1 gene expression on prostate cancer cell growth response to vitamin D₃.

A. qRT-PCR analysis of CYP24A1 mRNA levels in presence of 10nM Vitamin D₃ or vehicle (EtOH) in DU145 and PC3 transiently transfected with CYP24A1 siRNA or a non-targeting siRNA (Scramble). GAPDH served as the internal control. **B.** Immunofluorescent staining of CYP24A1 protein of DU145 and PC3 transiently transfected with CYP24A1 siRNA or a non-targeting siRNA (Scramble) and incubated in presence of Vitamin D₃ 10 nM. DAPI was used for cell nuclei visualization. **C-D.** Cell proliferation assays performed in presence of 10 nM or 100 nM calcitriol or ethanol after 6 days of treatment in DU145 (C) and PC3 (D) transiently transfected with CYP24A1 siRNA or non targeting siRNA (Scramble). The values represent the mean of at least three separate experiments. Significance was evaluated by ANOVA, * if P < .05; ** if P < .01; *** if P < .001.

(http://www.dharmacon.com/CatalogSearch/ConsolidatedSearch.aspx?searchTerm=cyp24a1&searchTarget=1591_+CYP24A1).

2.b. Selective inhibition of CYP24A1 Expression in Human Prostate Cancer Cell Lines: Effect on Vitamin D Action on Cell Proliferation: Using this approach, we were able to selectively inhibit CYP24A1 expression in prostate cancer cell lines that are resistant to vitamin D₃ using RNA interference. The relative mRNA levels of CYP24A1 were efficiently knocked down by siRNA in absence or presence of vitamin D₃ and asked whether the alteration of CYP24A1 gene expression would enhance growth inhibition by vitamin D₃ (Figure 5A). When

compared to cells transfected with the non-targeting siRNA, the abrogation of expression by CYP24A1 siRNA in PC3 cells was about 80% in constitutive conditions and 74 % in presence of 10 nM vitamin D₃ (Figure 5A). The knockdown of CYP24A1 mRNA translated into reduced levels of CYP24A1 protein, as reflected by the attenuation of the punctuated staining seen in cells transfected with CYP24A1 siRNA compared to control cells transfected with scramble (Figure 5C). To assess the cellular consequences of siRNA-mediated silencing of CYP24A1 gene expression, proliferation assays were performed. While PC3 and DU145 cells were not responsive to the growth inhibition mediated by vitamin D₃ (Figures 5D and 5E), CYP24A1 siRNA significantly enhanced the anti-proliferative action of vitamin D₃ (Figures 5D and 5E). Transient transfection of CYP24A1 siRNA also produced a significant enhancement of the growth inhibitory action of calcitriol in the LNCaP cell line which is responsive to vitamin D₃. This study shows the role of vitamin D₃ as a paracrine hormone to inhibit the growth of the prostate and provide a rationale basis to the use of specific inhibitors of CYP24A1 to enhance vitamin D-based therapies for prostate cancer management.

3. Selective Inhibition of CYP24A1 Enhances The Anti-Proliferative Effect of 1,25(OH)₂D₃ in Stably Transduced Prostate Cancer Cell Lines: To assess the impact of a sustained expression of CYP24A1 siRNA on the growth abrogation mediated by vitamin D₃, we generated LnCAP, DU145 and PC3 stable cell lines expressing CYP24A1shRNA or non-targeting shRNA. The random integration into the genome of the vector-based shRNA led to a significant knock down of the CYP24A1 expression in presence or absence of calcitriol (Figures 6A and 6C). Indeed, when compared to cells with non-targeting shRNA, relative CYP24A1 mRNA levels were reduced by 75% in PC3 and 85% in DU145 in presence of ethanol, and the knockdown was about 90% for PC3 and 60% for DU145 in presence of vitamin D₃. In these clonal conditions, PC3 showed an improvement of its growth response to vitamin D₃, reaching significance at 100 nM (Figure 6B). In DU145, a significant reduction of cell proliferation was already achieved at 10 nM of vitamin D₃ (Figure 6D). Hence, the prolonged abrogation of CYP24A1 gene expression enhanced the 1,25(OH)₂D₃-mediated growth inhibition in prostate cancer cells.

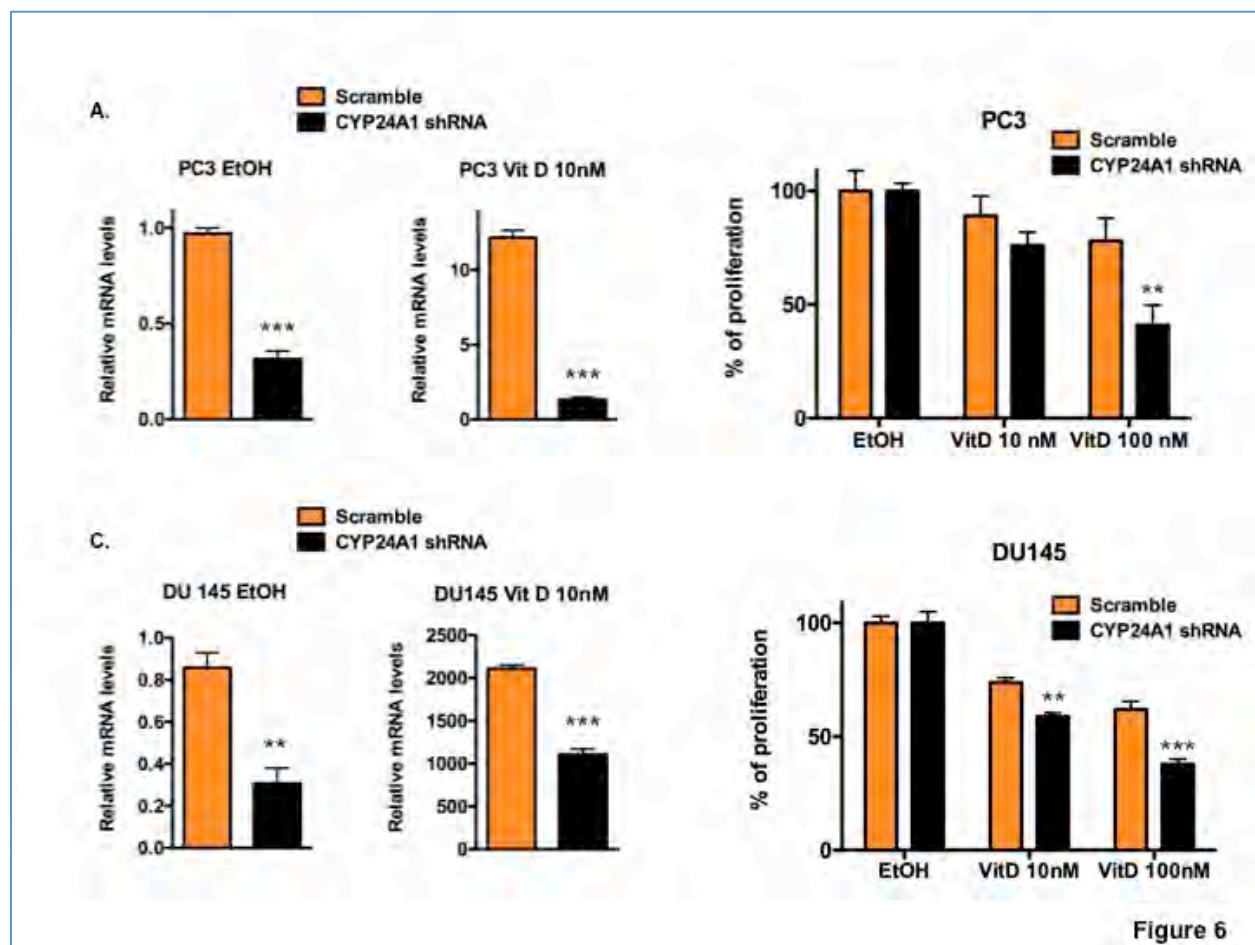


Figure 6: Generation of stable clones expressing shRNA and impact of sustained alteration of CYP24A1 gene expression on prostate cancer cell growth response to vitamin D₃.

A and C. qRT-PCR analysis of CYP24A1 mRNA levels in presence of 10nM Vitamin D₃ or vehicle (EtOH) in PC3 (A) and DU145 (C) stably transfected with CYP24A1 shRNA or a non targeting shRNA (Scramble). GAPDH served as the internal control. **B and D.** Cell proliferation assays performed in presence of 10 nM or 100 nM calcitriol or ethanol for 6 days in stable PC3 (B) and DU145 (D) expressing CYP24A1 shRNA or non targeting shRNA (scramble). The values represent the mean of at least three separate experiments. Significance was evaluated by ANOVA, * if $P < .05$; ** if $P < .01$; *** if $P < .001$.

This work shows that CYP24A1 was overexpressed in patients with prostate cancer, particularly in high-stage cases. In addition to highlight the endogenous anti-cancer action of vitamin D₃ in human prostate, this deregulation may be a predictive marker of vitamin D₃

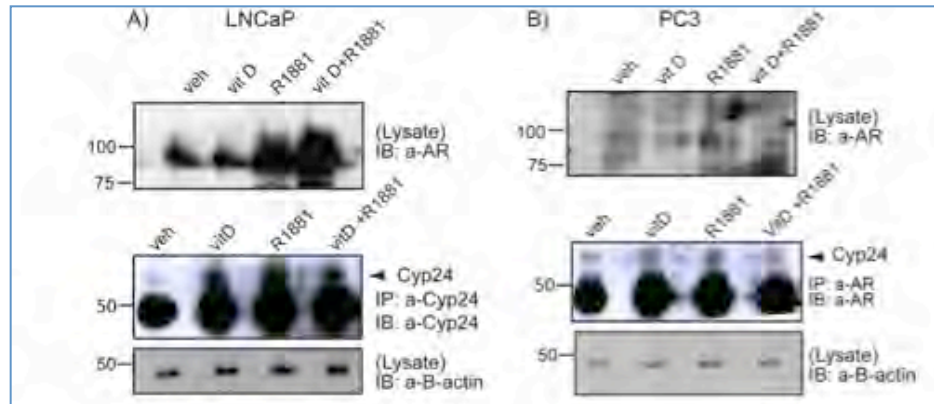


Figure 7 Expression of AR and CYP24A1 in androgen-dependent and independent cell lines. Cells were plated in 6 well plate to 60% confluence and treated with 0.1% ethanol, 10nM 1 α 25(OH)₂ D₃ and /or 10nM R1881 for 48 hr. Cell lysates were immunoprecipitated with anti CYP24A1 and probed for AR, CYP24A1 and β -actin antibody.

clinical efficacy in patients with advanced prostate cancer as an adjuvant therapy. Our findings also provide for the first time, a proof of principle that targeting of CYP24A1 by siRNA, a more selective and specific approach and as a result, a potentially less toxic alternative, is efficient to enhance the anti-proliferative action of vitamin D₃ in prostate cancer cells, offering a promising combination therapy for vitamin D-based therapies for advanced prostate cancer.

4. Androgen Administration Enhances the Growth Inhibitory Effect of Vitamin D₃: We studied the actions of androgen (alone or together with vitamin D₃) administration on cell using WST-1 colorimetric assay (Fig.7). In LNCaP (expresses a mutant AR and displays androgen-regulated growth) and PC3AR cells (stably transfected expressing the androgen receptor (AR)), R1881 (a synthetic androgen) administration caused 43% decrease in the cell growth. Similarly 1,25(OH)₂D₃ simultaneously inhibited LNCaP cell growth by 33%. The combined effect of R1881 and Vitamin D₃ were more pronounced, showing almost a 50% inhibition by 72 hr. The synergistic effect of the androgens and Vitamin D₃ is more potent, exhibiting a strong anti proliferative effect in AR-dependent cells within 72 hrs. In contrast, AR-independent PC3 (lacking androgen receptor) was not significantly growth inhibited by R1881 and Vitamin D₃. LNCaP_AR cells (androgen receptor present but growth resistant to androgens) showed a pattern similar to LNCaP. The cell line lacking AR (PC3) was resistant to Vit D₃ or R1881 administration and this was reversed when androgen receptors were stably transfected (PC3AR). Studies have shown that DHT has a biphasic effect on LNCaP cells, at low doses (0.1nM- 1 nM), it has a growth stimulatory effect and at higher doses there is a gradual loss of the growth stimulatory action of DHT. Our study was done at 100nM R1881, where we see a growth inhibition.

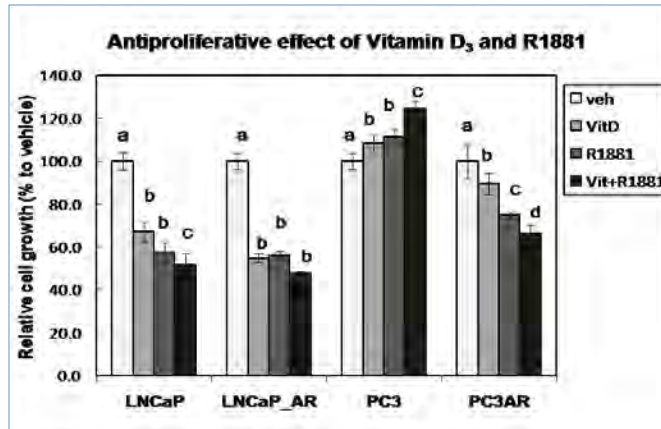


Figure 8 Effect of androgen on the growth inhibitory effect of 1,25(OH)₂D₃ in LNCaP, LNCaP_AR, PC3 and PC3AR cells. Cells were grown to 50% confluence, treated with 0.1% ethanol, 10nM 1,25(OH)₂D₃ and /or 100nM R1881 and harvested after 72 hr. Colorimetric cell proliferation assay by WST-1 reagent was used to measure the cell proliferation (n=6, 2 exp. replications). Two-way ANOVA shows statistically significant interaction ($p<0.05$) between 1,25(OH)₂D₃ and R1881 in LNCaP and PC3AR cell lines

In androgen-independent cell lines, PC3, DU145 and LNCaP_AR (C-42, a stable androgen independent cell line), the expression of CYP24A1mRNA induced by 1,25(OH)₂D₃ was not significantly inhibited by androgens. Thus, androgens down-regulate CYP24A1 gene expression in androgen-dependent cell lines, thereby enhancing the antiproliferative functions of Vitamin D₃. Western blot analysis demonstrated a robust expression of AR in LNCaP and PC3AR cells (not shown) with no expression in PC3 cells (Fig.7). AR was stabilized and showed a graded response with induction of 10nM 1,25(OH)₂D₃ and 10nM R1881, whereas, in PC3, DU145 and LNCaP_AR cells there was little or no expression of AR. CYP24A1 protein is over-expressed in all human prostate cancer cell lines. CYP24A1 expression is enhanced with the administration of 10nM 1,25(OH)₂D₃ in both AR-dependent (LNCaP) and AR independent (PC3) cell line.

R1881 administration to androgen-responsive prostate cancer cells enhances the

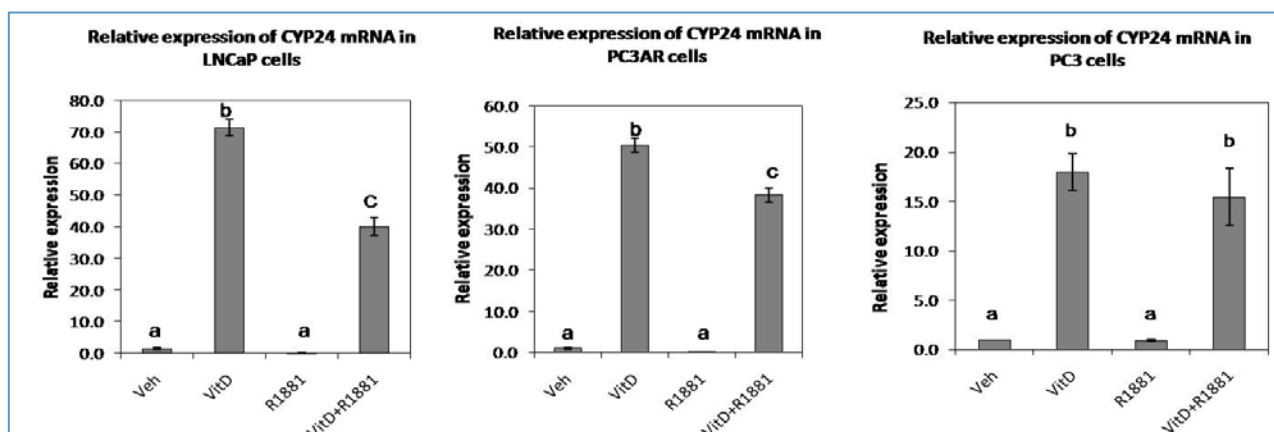


Figure 9 Effect of androgen on the level of 24-hydroxylase mRNA in LNCaP (A), PC-3 AR (B) and PC3 (C) cells. Cells were treated with 0.1% ethanol, 10nM 1,25(OH)₂D₃ and /or 10nM R1881 for 48 hr. Total RNA was isolated and CYP24A1mRNA estimated by real-time RT PCR (n=3). Two-way ANOVA shows statistically significant interaction between 1,25(OH)₂D₃ and R1881 in LNCaP and PC-3 AR cell lines.

5. Effect of Synthetic Androgen (R1881) on CYP24A1 and Androgen Receptor Gene Expression: Androgen-dependent and -independent human prostate cancer cell lines (as described above) were pretreated with 10nM 1,25(OH)₂D₃ in absence or presence of 10nM R1881 and were harvested after 48 hr. As expected (18,21), vitamin D₃ induction significantly enhanced the expression of CYP24A1mRNA with the highest level of induction being found in

the LNCaP cell line (Fig.8). Similar results were reported by others. In contrast, pre-incubation with 10nM R1881 together with 10nM 1,25(OH)₂D₃ significantly suppressed the expression of CYP24, indicating that R1881 at physiological concentration protects Vitamin D₃ from catabolism. In androgen-independent cell lines, PC3, DU145 and LNCaP_AR (C-42, a stable androgen independent cell line), the expression of CYP24A1mRNA induced by 1,25(OH)₂D₃ was not significantly inhibited by androgens. Thus, androgens down-regulate CYP24A1 gene expression in androgen-dependent cell lines, thereby enhancing the antiproliferative functions of Vitamin D₃. Western blot analysis demonstrated a robust expression of AR in LNCaP and PC3AR cells (not shown) with no expression in PC3 cells (Fig.7). AR was stabilized and showed a graded response with induction of 10nM 1,25(OH)₂D₃ and 10nM R1881, whereas, in PC3, DU145 and LNCaP_AR cells there was little or no expression of AR. CYP24A1 protein is over-expressed in all human prostate cancer cell lines. CYP24A1 expression is enhanced with the administration of 10nM 1,25(OH)₂D₃ in both AR-dependent (LNCaP) and AR independent (PC3) cell line.

antiproliferative activity of Vitamin D₃ (Fig.9) and protects Vitamin D₃ from inactivation by suppressing CYP24A1 expression. **Results suggest that androgen ablation actually converts the prostate cancer cells to a state of vitamin D resistance.**

6. Resveratrol Potentiates The Anti-Proliferative Effects of Calcitriol by Enhancing The Transcriptional Activity of the

VDR: Resveratrol (3,4',5-trihydroxy-*trans*-stilbene) is a polyphenol highly enriched in grapes, berries, peanuts and other dietary sources. Resveratrol exerts inhibitory effects on the initiation, promotion, and progression of carcinogenesis by modulating signal transduction pathways that control cell division and growth, apoptosis, inflammation, angiogenesis, and metastasis. Treatment with low-dose resveratrol and calcitriol in combination drastically inhibits cell proliferation. resveratrol used at low doses (5 μ M) potentiates the anti-proliferative effects of calcitriol. While resveratrol (5 μ M) or calcitriol (1-10 nM) alone were not effective in suppressing the growth of the prostate cancer cells, DU145, combined treatment with resveratrol and calcitriol drastically inhibited cell proliferation as assessed by MTT proliferation assays (Figure 10).

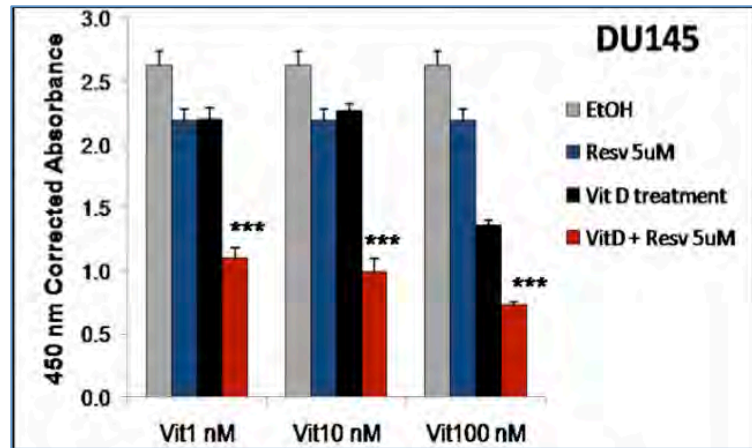


Figure 10. Effect of resveratrol on the growth inhibitory effect of 1,25(OH)₂D₃ in DU145 cells. Cells were treated with 0.1% ethanol, 1, 10nM or 100 nM 1,25(OH)₂D₃ and resveratrol (5 μ M) and harvested after 6 days. Colorimetric cell proliferation assay by WTT reagent was used to measure the cell proliferation (Values represent the mean of at least three separate experiments). One-way ANOVA shows statistically significance of the interaction ($p < 0.001$) between 1,25(OH)₂D₃ and resveratrol in DU145 cells.

Genes involved in the control of cell proliferation such as GADD45 (growth arrest and DNA-damage-inducible gene), IGFBP3 (insulin growth factor binding protein 3) and CDKN1A that encodes the cyclin dependent kinase inhibitor p21, were transcriptionally activated by 1 α ,25(OH)₂D₃. Their levels of expression increased with resveratrol as well. However, when the cells were treated with both resveratrol and calcitriol, their levels of mRNA were significantly increased compared to cells subjected to single treatment (Figure 11).

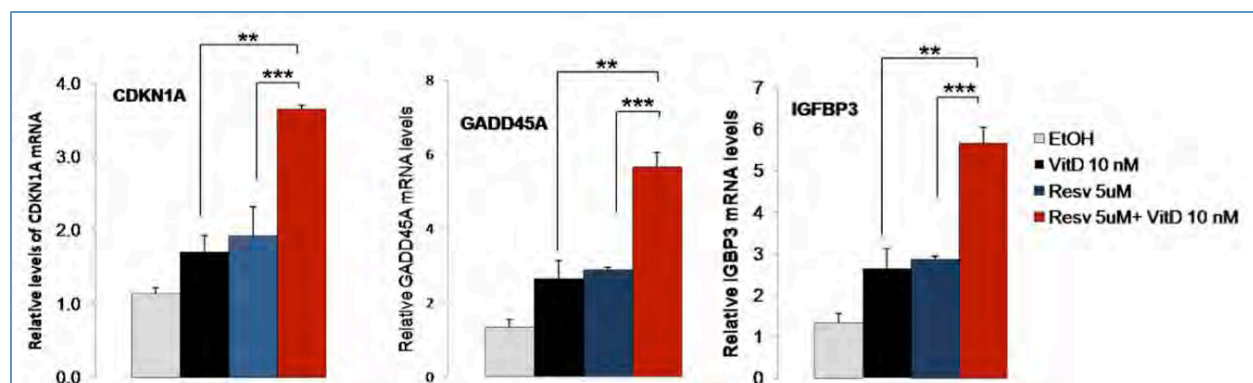


Figure 11 Synergistic transcriptional effect of resveratrol and calcitriol on vitamin D responsive genes. Taqman qRT-PCR analysis of CDKN1A (p21), GADD45A and IGFBP3 mRNA levels in DU145 cells treated for 24 h with 10 nM Vitamin D₃ and/or resveratrol (5 μ M) or vehicle (EtOH). GAPDH served as the internal control. ** $p < 0.01$, *** $p < 0.001$.

However, the transcriptional synergistic effect on the vitamin D responsive genes could not be

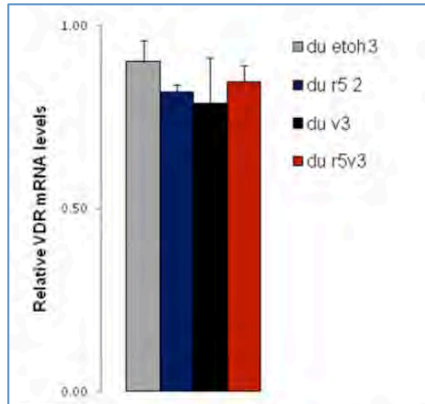


Figure 13 Gene expression levels of VDR are not affected by combined treatment of resveratrol and vitamin D

due to the increase of mRNA levels of the vitamin D receptor (VDR) since these agents used alone or in combination did not induce VDR gene expression (Figure 13). Potentiation occurs through a VDRE element as shown by luciferase assays (Figure 14). Indeed, a synergistic effect was obtained when both resveratrol and calcitriol were used on the VDRE. To define the molecular mechanisms underlying this potentiation, we tested whether the interaction of VDR with coactivators is enhanced in presence of both components. One important interacting cofactor with VDR is the p160 family member, SRC-1. Transfection with an SRC-1 expression vector enhanced the transcriptional activity on a VDRE in presence of resveratrol and calcitriol.

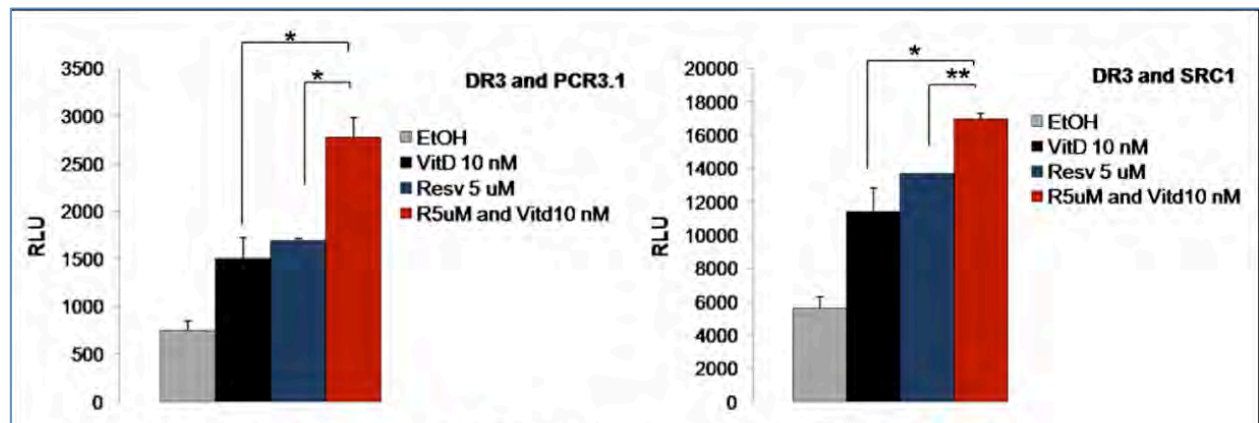


Figure 14 Luciferase assay using VDRE luciferase reporter in the presence or absence of 10 nM Vitamin D and/or 5 μ M resveratrol. Left pane shows co-transfection with an empty vector. Right panel shows co-transfection with an SRC-1 expression vector

Specific Aim #2 To Enhance Vitamin D inhibition of prostate cancer proliferation by CYP24A1siRNA in vivo.

We generated stable CYP24A1shRNA expressing prostate cancer cell lines (section C.3) and enhanced the anti-proliferative effect of calcitriol when compared to control cells expressing non-targeting shRNA. The control shRNA (a commercially available general purpose 21 mre, 48% GC) has no homology to human, mouse or rat mRNA. SCID mice were maintained on a vitamin D₃ deficient diet containing 0.5% calcium (#5826-CI, Purina) for 2 weeks prior to use. The recipient mice were anesthetized and injected intraprostatically with 1×10^6 stably transfected prostate cancer cells. This cell concentration achieves consistent local tumor growth within 7 days of implantation. Two weeks after injection, mice were separated into groups of 8 animals each and treated with control- sesame oil with 2% ethanol or 0.5 μ g/kg 1,25(OH)₂D₃ (in sesame oil with 2% ethanol) every other day by oral gavages with a 20 gauge intragastric feeding tube.

In these studies, MR images were obtained using a Bruker Biospin Pharmascan 7.0T spectrometer (Bruker Biospin, Billerica, MA) in the Mouse Phenotyping Core at Baylor College of Medicine so that we could non-invasively follow xenograft tumor growth over time. The average size of the prostate prior to intraprostatic injection of prostate cancer cells was 24 mm³.

In animals receiving DU145 cells stably transfected with the CYP24A1siRNA (DU41 cells), the combined volume of the prostate gland and tumor is 14.7 mm^3 at D2 and decreased in size to 3.07 mm^3 on D18 of vitamin D treatment. There are a total of 8 animals (out of 40 total in the study; $n = 2$ mice / treatment group) that underwent repeated MR imaging in-order-to monitor tumor growth. In the remaining mice (as well as the imaged mice), the tumors were removed at day 60 for analysis. Baseline volume (D0) of the prostate gland was determined and averaged.

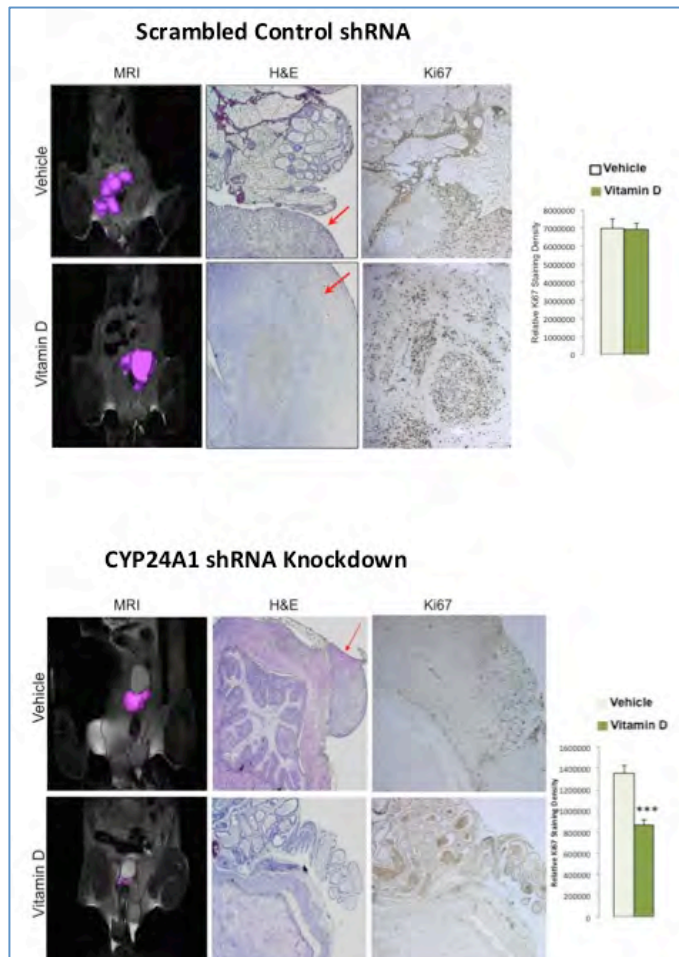


Figure 15 Vitamin D inhibition of human prostate cancer xenografts after CYP24A1 knockdown. CYP24A1 knockdown (lower panels) and scrambled shRNA (top panels) in DU145 cells injected orthotopically in the prostate using male SCID mice receiving $1, 25(\text{OH})_2\text{D}_3$. DU145 cells stably transduced with CYP24A1siRNA (lower) or scrambled controls (upper) (1×10^6 DU41 cells) were injected intraprostatically after surgical exposure of the dorsal prostate gland. Mice are treated with $0.5 \mu\text{g}/\text{kg}$ $1,25(\text{OH})_2\text{D}_3$ (in sesame oil with 2% ethanol) every other day by oral gavage. Purple indicates tumor. MRI imaging of SCID mice at 60 days after xenograft injection (left panels). MRI images were obtained in the Mouse Phenotyping Core at Baylor College of Medicine. The images are T2-weighted (TR=3000ms and TE=36.61ms). The slice thickness is 0.5 mm (no interslice gaps) and the field of view (FOV) is 40 mm x 40 mm. The right panels show H&E staining of the prostate cancer xenograft and further right is shown the Ki67 staining. Ki67 staining shows cell growth inhibition by $1,25(\text{OH})_2\text{D}_3$ only in the cancer cells after CYP24 knockdown. The bar graphs show the relative degree of proliferation based on the Ki67 staining.

Figure 15 simply shows representative results at day 60 of treatment expressed as tumor volume calculated by MRI. The tumors grew at similar rates in the presence or absence of calcitriol with control (scrambled) shRNA (upper figures) cells and with CYP24 shRNA and vehicle only (lower panel). Importantly, the mice with the tumor xenograft with CYP24 knockdown showed highly significant inhibition of tumor growth over the same time period. This growth inhibition was also evident when the tumors histology was assessed with decreased ki67 staining apparent which correlates with proliferative index (right panels and bar graphs).

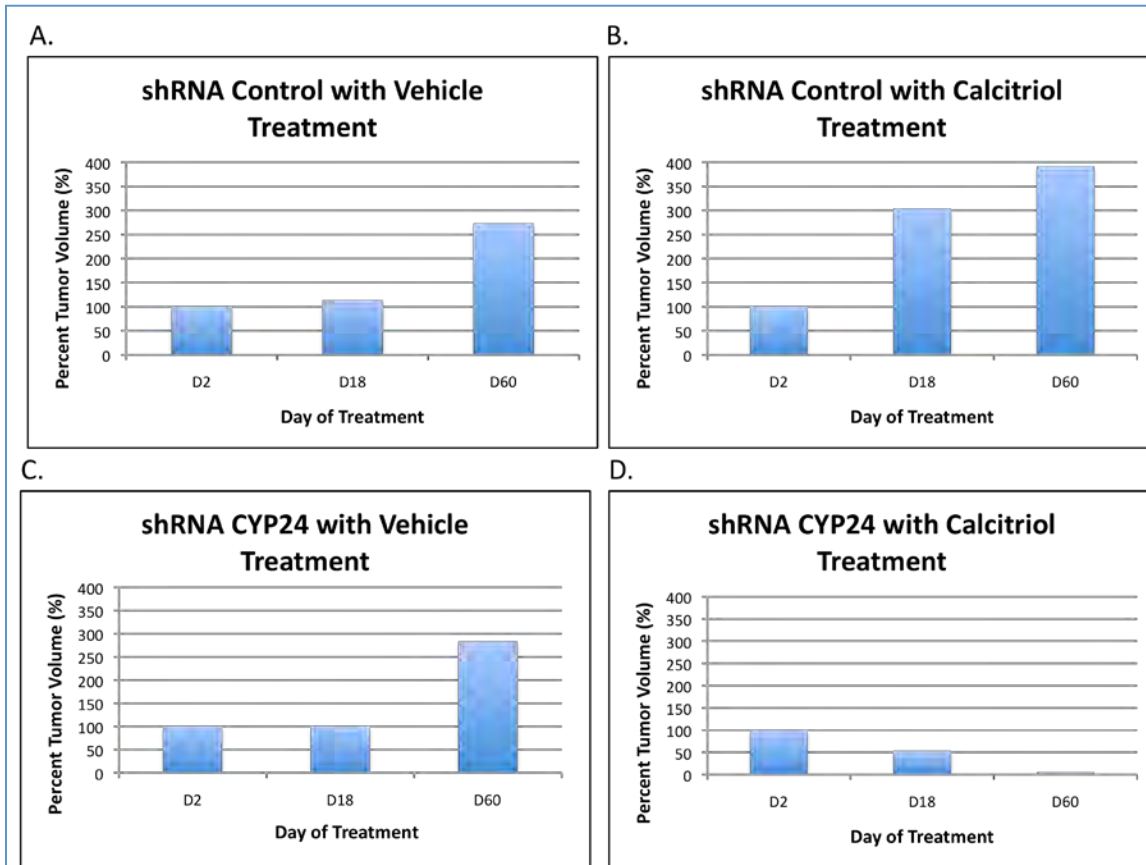
Figure 16 shows the tumor volumes of representative individual mice over time beginning with treatment day 2. It is apparent that that control xenograft cells (scrambled shRNA) continue to grow over the study period ending at 60 days whereas the growth of the shRNA CYP24A1 cells was markedly inhibited by Vitamin D_3 over the 60-day study period.

These studies provide evidence that CYP24A1 knockdown in human prostate cancer cells tested in a xenograft model enhances the growth inhibiting/apoptotic actions of Vitamin D_3 administration in prostate cancer. We performed additional histological and phenotypic

assays to further define the effect of CYP24A1 knockdown on prostate cancer cell growth. Once total tumor burden was 1.5 cm, the mice were anesthetized and cardiac puncture performed to

collect serum. Tumor growth and sites of metastasis especially pulmonary and retroperitoneal metastases, commonly observed in advanced prostate cancer were assessed. Average tumor size and weight were measured and tumor volume calculated using the equation: tumor volume (cm³) = 0.523 x [length (cm) x width² (cm²)]. Specimens were fixed in formalin and embedded in paraffin for hematoxylin-eosin staining or for proliferation markers immunostaining

Figure 16: Graphs A-D show tumor volumes over the course of treatment as a percentage of the starting tumor volume at treatment day 2. Each graph shows data from representative treatment groups in this orthotopic xenograft experiment where mice were injected intraprostatically with DU145 prostate cancer cells stably transduced with either CYP24 targeted or control shRNA. The mice were then given treatments every other day with calcitriol or vehicle.



(Proliferating Cell Nuclear Antigen) and apoptosis using the TUNEL assay. Specimens were cryopreserved for further protein and/or RNA analysis of proliferation and apoptosis markers. Serum collected by cardiac puncture were used for calcium measurement to detect hypercalcemia (normal calcium values being about 8 mg/dl) and kidney sections were stained for calcium using the von Kossa stain or Alizarin Red S.

Major Findings of This Final Progress Report Studies are:

- a) **CYP24A1(RNA and Protein) is over-expressed in prostate cancer and increases with TNM**
- b) **CYP24A1siRNA blocks CYP24A1expression and caused vitamin D₃ resistant prostate cancer to become vitamin D₃ sensitive and to once again be growth inhibited by 1,25(OH)₂D₃.**
 1. **Growth inhibition is seen in vivo and in vitro**

2. *This is a drugable pathway (for future studies)*

c) *Androgens enhance the antiproliferative action of vitamin D₃ in prostate cancer by inhibiting CYP24A1 expression*

d). *Resveratrol markedly enhanced vitamin D₃ action working through SRC-1 and VDR*

Publications Resulting from these Investigations:

One paper (enclosed) is submitted and under review at Cancer Research. This paper includes the work in Specific Aim #1 on the expression of CYP24A1 in human prostate cancer and prostate cancer cell lines, evidence of reversible vitamin D resistance by CYP24A1 knockdown and the production of stably transfected cell lines. This manuscript is included with this report.

Three other manuscripts are nearing completion for submission: The second manuscript focuses on the requirement for androgens and the androgen receptor for efficient vitamin D action. A third paper focuses on CYP24A1 expression in metastatic disease (using tissue arrays from warm autopsy specimens), xenograft studies of the stably transfected cell lines and the enhancement of vitamin D action in vivo in cells that are otherwise Vitamin D resistant. A fourth paper focuses on the use of resveratrol to modify vitamin D action in prostate cancer cell lines through an effect on SRC-1.

Selective Targeting of CYP24A1 as a Rational Approach for Vitamin D₃ Based Therapies in Human Prostate Cancer

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ABSTRACT

Purpose: Clinical trials to develop vitamin D₃-based therapies for advanced prostate cancer were hindered by the risk of hypercalcemia and the limited antitumor benefit. We aimed to define factors predictive of the tumor growth response to vitamin D₃ in patients with prostate cancer and specifically examine the role of its major catabolic enzyme, CYP24A1 in prostate tumorigenesis.

Experimental Design: Real-time PCR and immunostaining were performed using 36 benign and cancer biopsy samples and tissue microarrays with 112 prostate cancer cores derived from 56 patients. The functional role of CYP24A1 and the impact of selective inhibition of its expression on the anti-proliferative action of vitamin D₃ were examined in human prostate cancer cell lines.

Results: CYP24A1 mRNA was elevated in malignant human prostate tissues compared to benign lesions. High CYP24A1 protein levels were seen in poorly differentiated and highly advanced stages of prostate cancer ($P<0.0001$) and correlated with parallel increased staining of Ki67, a marker of proliferation ($P<0.0002$). While levels of CYP24A1 mRNA in prostate cancer cells inversely correlated with their respective growth response to vitamin D₃, abrogation of CYP24A1 expression using specific siRNA significantly enhanced the vitamin D₃-mediated growth inhibition.

Conclusions: Increased CYP24A1 expression may restrict the vitamin D₃-mediated growth inhibition in patients. The use of CYP24A1 siRNA renders cancer cells more sensitive to the anti-proliferative action of vitamin D₃ and could be used in combination with therapeutic doses of vitamin D₃ for prostate cancer treatment.

INTRODUCTION

Prostate cancer is the most commonly diagnosed malignancy in men. Treatments include radical prostatectomy followed by radiation or hormone ablation therapy. Unfortunately, durable clinical responses are not always obtained and as prostate carcinoma progresses, systemic metastasis to distant areas lead to patient morbidity and mortality. Therefore, development of effective therapeutic strategies are needed for the management of castrate-resistant prostate cancer. One promising alternative is the use of the hormonal form of vitamin D₃, 1,25-dihydroxyvitamin D₃ (1,25(OH)₂D₃) or calcitriol, which exerts pro-differentiative, anti-proliferative, anti-angiogenic and pro-apoptotic effects on prostate cancer cells *in vitro* and *in vivo* (see for review (1)). Phase I and II trials of calcitriol, either alone or in combination with other therapeutic agents have been conducted in patients with castrate-resistant prostate cancer (2-8). However, the clinical use of vitamin D₃ based therapies was hampered by the risk of hypercalcemia and/or inadequate design for the optimal administration of calcitriol.

In order to improve the clinical efficacy of exogenous vitamin D₃ in the treatment of prostate cancer, it may be useful to define predictive factors for the growth suppressive response of vitamin D₃ in patients. In studying the action of 1,25(OH)₂D₃ in prostate cancer, it is essential to examine the potential pathways that control local tissue levels of 1,25(OH)₂D₃. The cytochrome P450 enzyme, 24-hydroxylase, encoded by CYP24A1 is central to the catabolism of 1,25(OH)₂D₃. While its constitutive expression levels are low, CYP24A1 is strongly induced by 1,25(OH)₂D₃ administration to produce less active vitamin D metabolites. Overexpression of CYP24A1 was reported in various human tumors including colon (9-11), cervical (12), ovarian (9, 12), esophageal (13), lung (14) and basal cell carcinomas (15). Changes of the expression or activity of vitamin D₃ catabolic enzyme CYP24A1 may affect the bioavailability of 1,25(OH)₂ vitamin D₃ at the tumor site and abrogate the anti-proliferative action of vitamin D₃. This may lead to failure of any vitamin D₃ - based therapy in patients. Therefore, determining local CYP24A1 expression levels would allow for individualized treatments and higher clinical efficacy of exogenous vitamin D₃.

To date, no study examined the gene expression levels of endogenous CYP24A1 in the human prostate. In this present article, we evaluated the *in situ* expression of CYP24A1 in human

adenocarcinomas and benign prostate lesions. We analyzed its possible association with tumor cell proliferation and tested in human prostate cancer cell lines, whether the inhibition of CYP24A1 expression by RNA interference, could enhance the abrogating vitamin D- mediated growth control.

MATERIALS AND METHODS

Cell lines and chemicals

Human prostate cancer cell lines (LnCAP, PC3 and DU145) were obtained from the American Type Culture Collection (ATCC, Rockville, MD, USA) and grown in RPMI, DMEM or MEM containing 10% fetal bovine serum, respectively. All cells were maintained in a humidified 37°C incubator with 5% CO₂. Calcitriol (Sigma-Aldrich, St. Louis, MO, USA) was reconstituted in 100% ethanol, stored at -20°C and protected from light.

Transfection with small interfering RNA

Cells were plated in 6-well plates (1×10^5 /well) for RNA extraction or in 96 well plates ($1-1.5 \times 10^3$ /well) for cell proliferation assays. The next day, cells were transfected for 24 hours with 40 nM of nonspecific (Scramble) or siRNA for human CYP24A1 (Dharmacon, Lafayette, CO, USA) using the Lipofectamine 2000 reagent (Invitrogen, CA, USA) according to the manufacturer's procedure. Following transfection, the cells were treated with either vehicle (0.1% v/v ethanol) or calcitriol (10-100 nM), for 24 h (RNA extraction) or 6 days (for cell proliferation).

Cell proliferation assays

Briefly, $1-1.5 \times 10^3$ cells per well were seeded into 96-well plates and treated with or without CYP24A1 siRNA for 24 h, then cultured in the presence or absence of 1,25(OH)₂D₃ at concentrations ranging from 1 nM to 100 nM. Viable cell number was determined after 3, 6 or 9 days of treatment using the WST1 Cell proliferation (Roche Applied Science, IN, USA) according to the manufacturer's instructions. The plates were read using a microplate spectrophotometer at a wavelength of 450 nm and corrected to 630 nm. Each independent experiment was performed at least three times.

Creation of stable cell lines harboring CYP24A1 shRNA

To generate stable prostate cancer cells expressing human CYP24A1 or non-targeting shRNA, cells were incubated with lentiviral transduction particles producing a non-target control or human

CYP24A1 shRNA (Sigma-Aldrich, St. Louis, MO, USA), in presence of polybrene, according to the manufacturer's protocol. Stable clones were selected with 1 µg/mL puromycin for three weeks.

Quantitative RT-PCR analysis of CYP24A1 gene expression

Total RNA was purified using the RNeasy Kit including an optional DNase I treatment according to the manufacturer's instructions (Qiagen, Valencia, CA, USA). cDNA was prepared from 500 ng of total RNA by reverse transcription. Patients' cDNA were prepared from well-documented cancer biopsy samples, normalized and assembled into ready-to-use gene expression panels (Origene, MD, USA). The supplier provided clinicopathological information for each patient. Real-time PCR was performed with TaqMan PCR Master Mix on an ABI StepOnePlus Realtime PCR System (Applied Biosystems, USA). PCR conditions were: 50°C for 2 min, 94°C for 2 min, followed by 40 cycles of 94°C for 15 s and 60°C for 30 s. For each experimental sample, the relative abundance value was normalized to the value derived from the endogenous control (beta-actin or GAPDH) of the same sample. Relative mRNA levels were quantified by the comparative $\Delta\Delta CT$ method.

CYP24A1 Immunofluorescence

Non-confluent prostate cells were fixed in paraformaldehyde 4% for 5 min on ice. Labeling with CYP24A1 antibody (Santa-Cruz, CA USA) was carried out in the presence of 0.1% Triton X-100, followed by Alexa Fluor 488 goat anti-rabbit IgG secondary antibody. Cells were counterstained with 4',6-diamidino-2-phenylindole (DAPI) for cell nuclei visualization.

Tissue Microarrays and CYP24A1 immunohistochemical detection

Tissue microarrays containing 112 prostate cancer cores, derived from 56 patients with localized and metastatic prostate cancer disease and Gleason scores ranging from 4 to 10 (Folio, OH, USA) were used in this study. Slides of paraffin-embedded adenocarcinomas samples were deparaffinized and dehydrated. Antigen retrieval was performed by heat inactivation in 0.1 M sodium citrate for 30 min. CYP24A1 (Santa Cruz, CA, USA) or Ki67 (DAKO, CA USA) primary antibodies were

used for immunodetection. The staining was performed using the avidin-biotin peroxidase system (ABC-peroxidase), and positive signals were visualized as brown precipitates using 3,3'-diaminobenzidine tetra-hydrochloride. Control staining was conducted by omission of the primary antibody. Hematoxylin was used for counterstaining. Two investigators, blinded to the data, performed light microscopy (Axioplan 2, Carl Zeiss, USA) and scored semi-quantitatively the quantity of protein expression in the whole section (0=none, 1= moderate, 2=strong). Scoring for semi-quantitative analysis for Ki67 staining was evaluated by the percentage of cells showing positively labeled nuclei: 0 (0-1% positivity), 1 (1-5 % positivity), 2 (5-10% positivity), 3 (10-100% positivity).

Statistical analysis

The data were obtained from at least two different experiments and are presented as mean± S.E.M. All statistical analyses were performed using the one-way or two-way analysis of variance (ANOVA). Contingency tables with Chi-square analysis were done using GraphPad (La Jolla, CA, USA). Values were considered statistically significant if $P < .05$ (* if $P < .05$; ** if $P < .01$; *** if $P < .001$).

RESULTS

CYP24A1 Was Overexpressed In Patients With Prostate Cancer

To assess the mRNA levels of endogenous CYP24A1 in adenocarcinomas of the human prostate, cDNAs obtained from pathologist-verified human prostate biopsies representing different pathological stages of prostate cancer as well as benign prostatic hyperplasia (BPH) were analyzed by quantitative PCR. Comparison of the CYP24A1 gene expression profile showed statistically significant mRNA overexpression (4 fold; $P=0.03$, Wilcoxon rank-sum test) in prostate cancer samples ($n=29$) compared with benign tissue ($n=7$) (Figure 1A). Consistently, malignant tissues displayed higher levels of CYP24A1 protein than the paired cancer-adjacent normal tissues ($n=8$) (Figure 1B).

To gain insight into specific changes associated with cancer progression, we analyzed tissue samples from 112 prostate cancer cores, derived from 56 patients with localized and metastatic prostate cancer disease and Gleason scores ranging from 4 to 10. The percentage of immunoreactive cases with CYP24A1 was high (98% of cancer cores). However, the staining intensity varied between patients, ranging from intense immunoreactivity of the entire cytoplasm to more discrete granular staining. Importantly, high protein levels of CYP24A1 positively correlated with high-grade cancer (Figure 2; $P<0.0001$, Pearson's Chi-square) as well as with late stage tumor progression (Figure 2; $P<0.0001$, Pearson's Chi-square). Increased CYP24A1 staining intensity was indeed, frequently observed in cancer cores with Gleason scores higher than 7 and in lesions presenting with 3 or 4 TNM stages (Fig 2A-D).

Next, to examine whether increased CYP24A1 expression reflects increased tumor proliferation, we determined its relationship to the expression of the proliferation marker Ki67. High expression of CYP24A1 positively correlated with parallel increased staining of Ki67 in the same human prostate cancer tissues (Figure 3; $P<0.0002$, Pearson's Chi square). This observation supports the hypothesis that overexpression of CYP24A1 in prostate cancer cells may reduce the ability of 1,25-dihydroxyvitamin D₃ to inhibit proliferation.

Collectively, we demonstrate for the first time, the changes of expression of CYP24A1 in human patients with prostate cancer and highlight the positive correlation between elevated CYP24A1

protein levels and tumor progression and proliferation status. Our observations provide support for the potential key role of the vitamin D catabolizing enzyme CYP24A1 in human prostate cancer.

Inverse Correlation Between Basal Levels Of Human CYP24A1 mRNA And Growth Response To Vitamin D In Prostate Cancer Cells

Since CYP24A1 is the main catabolic enzyme determining the biological half-life of vitamin D₃, increased CYP24A1 expression in human prostate cancer tissues would presumably decrease the intra-tumor 1,25(OH)₂D₃ levels, effectively counteracting its anti-proliferative effects. As the steady-state levels of cellular vitamin D₃ in prostate tumor tissue of patients is difficult to measure, we analyzed the expression levels of endogenous CYP24A1 in three human prostate cancer cell lines (LnCAP, PC3 and DU145) and monitored their respective cell proliferation in presence of vitamin D₃. Consistent with the findings of previous reports on CYP24A1 enzymatic activity in these prostate cell lines (16), we found an inverse correlation between the constitutive cellular levels of CYP24A1 mRNA and the growth response to vitamin D₃ (Figures 4A-D). Indeed, LnCAP, with the lowest constitutive expression levels of CYP24A1 (Figure 4A), is most responsive to the anti-proliferative effect of calcitriol (Figure 4B). In contrast, PC3, which displays the highest basal levels of CYP24A1 (Figure 4A), is the most resistant to the inhibitory effect of calcitriol on cell growth (Figure 4D). Thus, the differential prostate cell response to calcitriol is due to their inherent constitutive gene expression levels of 1,25(OH)₂ vitamin D₃ catabolic enzyme, CYP24A1. These data support the role of vitamin D₃ as a paracrine hormone to inhibit the growth of the prostate and provide a rationale basis to the use of specific inhibitors of CYP24A1 to enhance vitamin D-based therapies for prostate cancer management.

Specific Inhibition Of CYP24A1 Expression By siRNA Enhanced The Anti-Proliferative Effect Of 1,25(OH)₂D₃ In Prostate Cancer Cells.

To investigate functional consequences of CYP24 overexpression, we used a siRNA-based approach. We chose to specifically target CYP24A1 expression with small interfering RNA (siRNA) in

human prostate cancer cells that are resistant to vitamin D₃ (PC3 and DU145) and examine whether the alteration of CYP24A1 gene expression would enhance the growth inhibition mediated by vitamin D₃. We first assessed by qRT-PCR, the efficacy of inhibition of CYP24A1 expression. The relative levels of CYP24A1 mRNA were efficiently knocked down by siRNA in absence or presence of vitamin D₃ (Figure 5A). When compared to cells transfected with the non-targeting siRNA, the abrogation of expression by CYP24A1 siRNA in DU145 and PC3 cells was about 90% and 80% in presence of vehicle (ethanol) and 60 and 74 % in presence of 10 nM vitamin D₃, respectively (Figures 5A-B). The knockdown of CYP24A1 mRNA translated into reduced levels of CYP24A1 protein, as reflected by the attenuation of the punctuated staining seen in cells transfected with CYP24A1 siRNA compared to control cells transfected with the scrambled control construct (Figure 5B). To assess the cellular consequences of siRNA-mediated silencing of CYP24A1 gene expression, proliferation assays were performed. While PC3 and DU145 cells were not responsive to the growth inhibition mediated by vitamin D₃ (Figures 2C and 2D), CYP24A1 siRNA significantly enhanced the anti-proliferative action of vitamin D₃ (Figures 5C and 5D). Taken together, knockdown of CYP24A1 gene expression by siRNA renders prostate cancer cells more sensitive to the growth-suppressive effect of vitamin D₃.

To assess the impact of a sustained expression of CYP24A1 siRNA on the growth abrogation mediated by vitamin D₃, we generated PC3 and DU145 cell lines stably expressing CYP24A1 shRNA or non-targeting shRNA. The random integration into the genome of the vector-based shRNA led to a significant knock down of the CYP24A1 expression in absence and presence of calcitriol (Figures 6A and 6C). Indeed, when compared to cells with non-targeting shRNA, relative CYP24A1 mRNA levels were reduced by 75% in PC3 and 85% in DU145 cells in presence of vehicle, and the knockdown was about 90% for PC3 and 60% for DU145 cells in presence of vitamin D₃. In these clonal conditions, PC3 cells showed an improvement of their growth inhibitory response to vitamin D₃, reaching significance at 100 nM (Figure 6B). In DU145 cells, a significant reduction of cell proliferation was already achieved at 10 nM of vitamin D₃ (Figure 6D). Hence, the prolonged abrogation of CYP24A1 gene expression enhanced the 1,25(OH)₂D₃-mediated growth inhibition in prostate cancer cells and essentially changed vitamin D₃ resistant cells into vitamin D₃ sensitive cell lines.

DISCUSSION

Our study is the first to compare the mRNA and protein levels of the major vitamin D₃ catabolizing enzyme, CYP24A1, in benign and cancerous human prostate tissues. Changes in the expression of this enzyme are associated with poor differentiation status and prognosis of other types of cancer including colon (9-11), cervical (12), ovarian (9, 12), esophageal (13), lung (14) and basal cell carcinomas (15). The chromosomal locus 20q13.2-20q13.3 encompassing CYP24A1 was found amplified in breast (17), ovarian (18), gastric (19, 20) and colorectal cancers (21). The increased expression of the rate-limiting catabolic enzyme, CYP24A1, is likely to restrict *in situ* 1,25(OH)₂D₃ growth abrogation activity, presumably by decreasing local 1,25(OH)₂D₃ levels. The significant correlation found between high CYP24A1 expression levels and increased proliferation rate in human prostate tumors supports this hypothesis. The fact that the suppression of CYP24A1 gene expression led to an enhanced 1,25(OH)₂D₃ anti-proliferative action in prostate cancer cells, provides an additional argument in favor of the *in situ* role of CYP24A1 in the control of prostate proliferation. Consistent with the notion that a selective growth advantage is given to the prostate tumor cells expressing high levels of CYP24A1, we found that overexpression of this catabolizing enzyme was associated with a more aggressive tumor behavior in prostate cancer. This latter observation is of clinical importance as CYP24A1 may represent a tissue biomarker of prostate tumorigenesis. Staining using a CYP24A1 immunohistochemistry-based test of prostate needle biopsies might be combined to existing tests in order to specifically stratify prostate cancer and predict the biological behavior of tumors. In addition to increase the accuracy in predicting prostate cancer disease outcome, CYP24A1 may serve to monitor treatment efficacy using exogenous vitamin D₃ as an adjuvant therapy for advanced prostate cancer.

Our findings indirectly point at the potential role for vitamin D₃ as a hormone acting locally to inhibit and perhaps to delay or prevent the progression to cancer. Epidemiological studies remain often-conflicting regarding an inverse association between sun exposure, serum levels of 25(OH)D, and risk of developing prostate cancer. Nevertheless, none of these studies assessed the intra-prostatic 1,25(OH)₂D₃ levels. By examining the tissue expression of the main enzyme responsible for

the breakdown of vitamin D₃, our present data shed light on the *in situ* dynamic importance of calcitriol bioavailability, thus enriching a large body of preclinical research and clinical trials evidence supporting the biological relevance of vitamin D in cell growth restriction and prostate cancer treatment (22).

This study offers a rational basis for the use of specific inhibitors of CYP24A1 in the management of prostate cancer. The inverse relationship between CYP24A1 expression and differentiation status in prostate cancer calls for the development of CYP24A1 selective inhibitors that can be used either as single entities delivered to the tumor site to increase or extend endogenous calcitriol function or in combination with exogenous doses of 1,25(OH)₂D₃ to achieve very localized high concentrations of vitamin D₃ resulting in optimal growth abrogation of advanced tumors with minimum systemic toxicity.

The use of cytochrome P450 inhibitors such as liarazole (23) and ketoconazole (24) or the phytoestrogen, genistein (25) synergistically enhanced the growth abrogation-mediated by vitamin D₃ by inhibiting the activity of CYP24A1 in prostate cancer cells. However, all these compounds lack selectivity and specificity and are likely to inhibit other cellular targets leading to long-term toxicity, thus rendering them incompatible or inefficient for clinical use. Ketoconazole and liarazole inhibit other P450 enzymes, including the enzymes involved in the steroidogenic pathways for testosterone, cortisol and aldosterone biosynthesis and may lead for instance, to adrenal insufficiency that would exacerbate the hypercalcemia of any vitamin D treatment. This present work explored the application of RNAi technology to selectively silence the expression of CYP24A1 in prostate cancer cells. RNAi-mediated degradation of homologous mRNA allows for a selective inhibition of key target molecules and offers a high specificity lacking in current drug treatments. Significant enhancement of the growth inhibitory action of vitamin D₃ achieved by combinatorial treatment with CYP24A1 siRNA holds a promise in the field of prostate cancer therapy. Nevertheless, significant obstacles, such as *in vivo* delivery, incomplete suppression of target genes need to be overcome before this technology can successfully be translated into the clinical arena.

This work shows that CYP24A1 was overexpressed in patients with prostate cancer, particularly in high-stage cases. In addition to highlight the endogenous anti-cancer action of vitamin

D₃ in human prostate, this dysregulation may be a predictive marker of vitamin D₃ clinical efficacy in patients with advanced prostate cancer as an adjuvant therapy. Our findings also provide for the first time, a proof of principle that targeting of CYP24A1 by siRNA, a more selective and specific approach and as a result, a potentially less toxic alternative, is efficient to enhance the anti-proliferative action of vitamin D₃ in prostate cancer cells, offering a promising combination therapy for vitamin D-based therapies for advanced prostate cancer.

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REFERENCES

1. Deeb KK, Trump DL, Johnson CS. Vitamin D signalling pathways in cancer: potential for anticancer therapeutics. *Nat Rev Cancer*. 2007;7:684-700.
2. Beer TM, Myrthue A, Garzotto M, O'Hara M F, Chin R, Lowe BA, et al. Randomized study of high-dose pulse calcitriol or placebo prior to radical prostatectomy. *Cancer Epidemiol Biomarkers Prev*. 2004;13:2225-32.
3. Beer TM, Ryan CW, Venner PM, Petrylak DP, Chatta GS, Ruether JD, et al. Double-blinded randomized study of high-dose calcitriol plus docetaxel compared with placebo plus docetaxel in androgen-independent prostate cancer: a report from the ASCENT Investigators. *J Clin Oncol*. 2007;25:669-74.
4. Flaig TW, Barqawi A, Miller G, Kane M, Zeng C, Crawford ED, et al. A phase II trial of dexamethasone, vitamin D, and carboplatin in patients with hormone-refractory prostate cancer. *Cancer*. 2006;107:266-74.
5. Liu G, Wilding G, Staab MJ, Horvath D, Miller K, Dresen A, et al. Phase II study of 1alpha-hydroxyvitamin D(2) in the treatment of advanced androgen-independent prostate cancer. *Clin Cancer Res*. 2003;9:4077-83.
6. Schwartz GG, Hall MC, Stindt D, Patton S, Lovato J, Torti FM. Phase I/II study of 19-nor-1alpha-25-dihydroxyvitamin D2 (paricalcitol) in advanced, androgen-insensitive prostate cancer. *Clin Cancer Res*. 2005;11:8680-5.
7. Tiffany NM, Ryan CW, Garzotto M, Wersinger EM, Beer TM. High dose pulse calcitriol, docetaxel and estramustine for androgen independent prostate cancer: a phase I/II study. *J Urol*. 2005;174:888-92.
8. Trump DL, Potter DM, Muindi J, Brufsky A, Johnson CS. Phase II trial of high-dose, intermittent calcitriol (1,25 dihydroxyvitamin D3) and dexamethasone in androgen-independent prostate cancer. *Cancer*. 2006;106:2136-42.

9. Anderson MG, Nakane M, Ruan X, Kroeger PE, Wu-Wong JR. Expression of VDR and CYP24A1 mRNA in human tumors. *Cancer Chemother Pharmacol.* 2006;57:234-40.
10. Bareis P, Bises G, Bischof MG, Cross HS, Peterlik M. 25-hydroxy-vitamin d metabolism in human colon cancer cells during tumor progression. *Biochem Biophys Res Commun.* 2001;285:1012-7.
11. Cross HS, Bises G, Lechner D, Manhardt T, Kallay E. The Vitamin D endocrine system of the gut--its possible role in colorectal cancer prevention. *J Steroid Biochem Mol Biol.* 2005;97:121-8.
12. Friedrich M, Rafi L, Mitschele T, Tilgen W, Schmidt W, Reichrath J. Analysis of the vitamin D system in cervical carcinomas, breast cancer and ovarian cancer. *Recent Results Cancer Res.* 2003;164:239-46.
13. Mimori K, Tanaka Y, Yoshinaga K, Masuda T, Yamashita K, Okamoto M, et al. Clinical significance of the overexpression of the candidate oncogene CYP24 in esophageal cancer. *Ann Oncol.* 2004;15:236-41.
14. Parise RA, Egorin MJ, Kanterewicz B, Taimi M, Petkovich M, Lew AM, et al. CYP24, the enzyme that catabolizes the antiproliferative agent vitamin D, is increased in lung cancer. *Int J Cancer.* 2006;119:1819-28.
15. Mitschele T, Diesel B, Friedrich M, Meineke V, Maas RM, Gartner BC, et al. Analysis of the vitamin D system in basal cell carcinomas (BCCs). *Lab Invest.* 2004;84:693-702.
16. Miller GJ, Stapleton GE, Hedlund TE, Moffat KA. Vitamin D receptor expression, 24-hydroxylase activity, and inhibition of growth by 1alpha,25-dihydroxyvitamin D3 in seven human prostatic carcinoma cell lines. *Clin Cancer Res.* 1995;1:997-1003.
17. Albertson DG, Ylstra B, Segraves R, Collins C, Dairkee SH, Kowbel D, et al. Quantitative mapping of amplicon structure by array CGH identifies CYP24 as a candidate oncogene. *Nat Genet.* 2000;25:144-6.
18. Tanner MM, Grenman S, Koul A, Johannsson O, Meltzer P, Pejovic T, et al. Frequent amplification of chromosomal region 20q12-q13 in ovarian cancer. *Clin Cancer Res.* 2000;6:1833-9.

19. Buffart TE, van Grieken NC, Tijssen M, Coffa J, Ylstra B, Grabsch HI, et al. High resolution analysis of DNA copy-number aberrations of chromosomes 8, 13, and 20 in gastric cancers. *Virchows Arch.* 2009;455:213-23.
20. Weiss MM, Snijders AM, Kuipers EJ, Ylstra B, Pinkel D, Meuwissen SG, et al. Determination of amplicon boundaries at 20q13.2 in tissue samples of human gastric adenocarcinomas by high-resolution microarray comparative genomic hybridization. *J Pathol.* 2003;200:320-6.
21. Lassmann S, Weis R, Makowiec F, Roth J, Danciu M, Hopt U, et al. Array CGH identifies distinct DNA copy number profiles of oncogenes and tumor suppressor genes in chromosomal- and microsatellite-unstable sporadic colorectal carcinomas. *J Mol Med.* 2007;85:293-304.
22. Schwartz GG. Vitamin D and intervention trials in prostate cancer: from theory to therapy. *Ann Epidemiol.* 2009;19:96-102.
23. Ly LH, Zhao XY, Holloway L, Feldman D. Liarozole acts synergistically with 1 α ,25-dihydroxyvitamin D₃ to inhibit growth of DU 145 human prostate cancer cells by blocking 24-hydroxylase activity. *Endocrinology.* 1999;140:2071-6.
24. Peehl DM, Seto E, Hsu JY, Feldman D. Preclinical activity of ketoconazole in combination with calcitriol or the vitamin D analogue EB 1089 in prostate cancer cells. *J Urol.* 2002;168:1583-8.
25. Swami S, Krishnan AV, Peehl DM, Feldman D. Genistein potentiates the growth inhibitory effects of 1,25-dihydroxyvitamin D₃ in DU145 human prostate cancer cells: role of the direct inhibition of CYP24 enzyme activity. *Mol Cell Endocrinol.* 2005;241:49-61.

FIGURE LEGENDS

Figure 1: Overexpression of CYP24A1 in human prostate cancer.

A. Determination of CYP24A1 gene expression levels in men with prostate cancer. TaqMan qPCR was performed on patients' cDNA, prepared from well-documented cancer biopsy samples, normalized and assembled into ready-to-use gene expression panels. Data were analyzed using the comparative C_T method with the values normalized to β -actin levels. **B.** Representative immunohistochemical detection of CYP24A1 revealing higher staining in malignant prostate tissue than in paired cancer-adjacent normal tissue. Magnification x40.

Figure 2: Correlation between high protein levels of CYP24A1 and high tumor grade and late stage of human prostate cancer. A-B. Distribution of the relative expression levels of CYP24A1 and Gleason score (A) and tumor stage (B) categories. Semi-quantitative scoring of the quantity of CYP24A1 expression in 112 prostate cancer cores, derived from 56 patients with localized and metastatic prostate cancer disease, was performed (score 1= moderate; score 2=strong). Cases are shown as percentages of total immunoreactive cancer cores on the y-axis. **C-D.** Immunostaining of CYP24A1 in representative adenocarcinomas of different grades. In **C.**, low grade lesions of prostate cancer showing moderate intensity of the staining of the catabolic enzyme (**a.** Gleason 2+2 and T2NxM0; **b.** Gleason 2+3 and T2NxM0; **c.** Gleason 2+3 and T2NxM0; **d.** Gleason 1+1 and T2bNxM0). In **D.**, intense staining in malignant prostate tissues presenting with high Gleason score and late stage tumor progression (**a.** Gleason 9 and T3bN0M0; **b.** Gleason 7 and T3bN0M0; **c.** Gleason 7 and T4N0M0; **d.** Gleason 9 and T3bN0M0). Magnification x40.

Figure 3: Distribution of the percentage of Ki67-positive cells between cancer cores expressing high (2) and moderate levels (1) of CYP24. Semi-quantitative scoring of the CYP24A1 staining (1= moderate, 2=strong) was independently performed by two investigators who were blinded to the data. Scoring for semi-quantitative analysis for Ki67 staining was evaluated by the percentage of cells showing positively labeled nuclei: 0 (0-1% positivity), 1 (1-5 % positivity), 2 (5-10% positivity), 3 (10-

100% positivity). Magnification x40.

Figure 4: Inverse correlation between endogenous levels of CYP24A1 mRNA and growth abrogation action of vitamin D₃.

A. qRT-PCR analysis of endogenous CYP24A1 mRNA levels in presence of 10nM Vitamin D₃ or vehicle (ethanol; EtOH) in LnCAP, DU145 and PC3 prostate cancer lines. GAPDH served as the internal control. **B-D.** Cell proliferation assays performed in presence of 10 nM or 100 nM calcitriol or ethanol after 3, 6 and 9 days of treatment in LnCAP (**B**), DU145 (**C**) and PC3 (**D**) (* if $P < .05$; ** if $P < .01$; *** if $P < .001$, ANOVA).

Figure 5: Effect of selective inhibition of CYP24A1 gene expression on prostate cancer cell growth response to vitamin D₃.

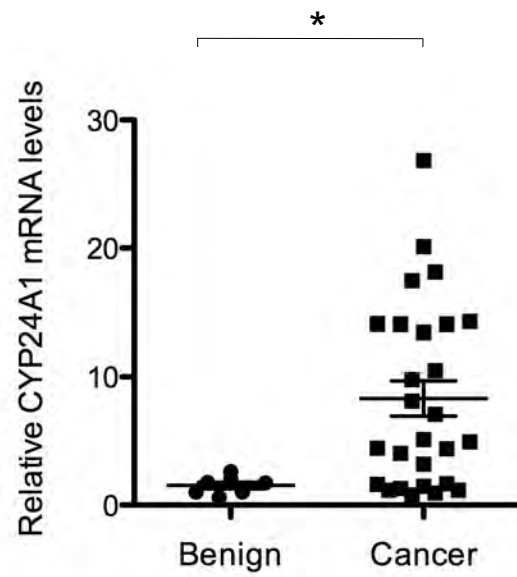
A. qRT-PCR analysis of CYP24A1 mRNA levels in presence of 10nM Vitamin D₃ or vehicle (EtOH) in DU145 and PC3 transiently transfected with CYP24A1 siRNA or a non targeting siRNA (Scramble). GAPDH served as the internal control. **B.** Immunofluorescent staining of CYP24A1 protein of DU145 and PC3 transiently transfected with CYP24A1 siRNA or a non-targeting siRNA (Scramble) and incubated in presence of Vitamin D₃ 10 nM. DAPI was used for cell nuclei visualization. **C-D.** Cell proliferation assays performed in presence of 10 nM or 100 nM calcitriol or ethanol after 6 days of treatment in DU145 (**C**) and PC3 (**D**) transiently transfected with CYP24A1 siRNA or non targeting siRNA (Scramble). The values represent the mean of at least three separate experiments. Significance was evaluated by ANOVA, * if $P < .05$; ** if $P < .01$; *** if $P < .001$.

Figure 6: Generation of stable clones expressing shRNA and impact of sustained alteration of CYP24A1 gene expression on prostate cancer cell growth response to vitamin D₃.

A and C. qRT-PCR analysis of CYP24A1 mRNA levels in presence of 10nM Vitamin D₃ or vehicle (EtOH) in PC3 (**A**) and DU145 (**C**) stably transfected with CYP24A1 shRNA or a non targeting shRNA (Scramble). GAPDH served as the internal control. **B and D.** Cell proliferation assays performed in

presence of 10 nM or 100 nM calcitriol or ethanol for 6 days in stable PC3 (**B**) and DU145 (**D**) expressing CYP24A1 shRNA or non targeting shRNA (scramble). The values represent the mean of at least three separate experiments. Significance was evaluated by ANOVA, * if $P < .05$; ** if $P < .01$; *** if $P < .001$.

A.



B.

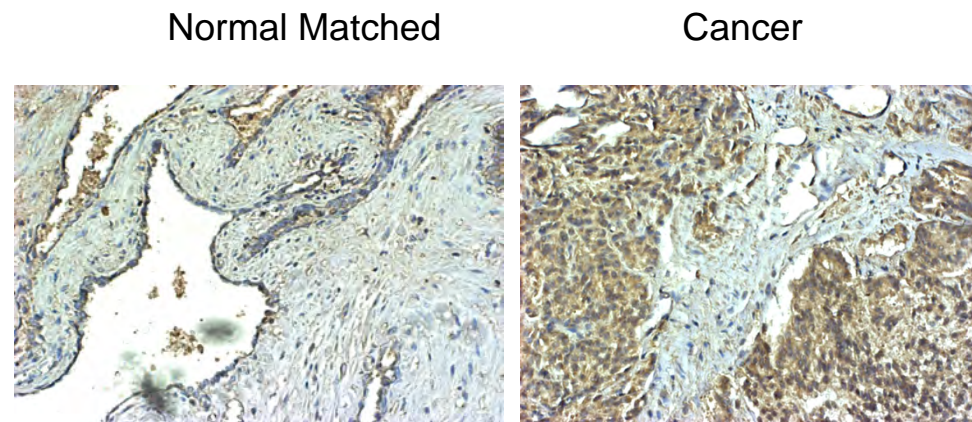
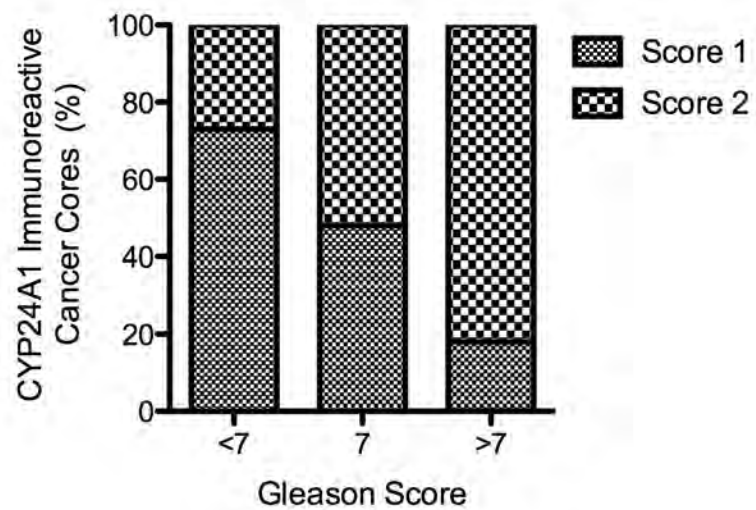
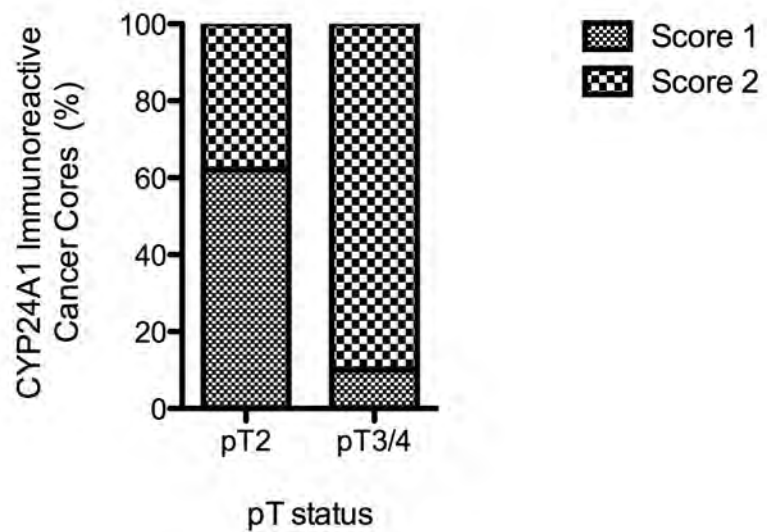


Figure 1

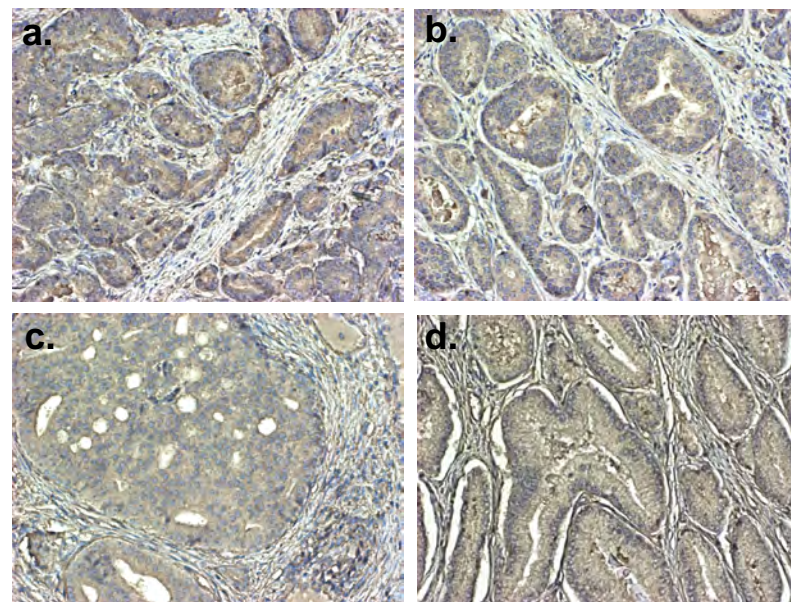
A.



B.



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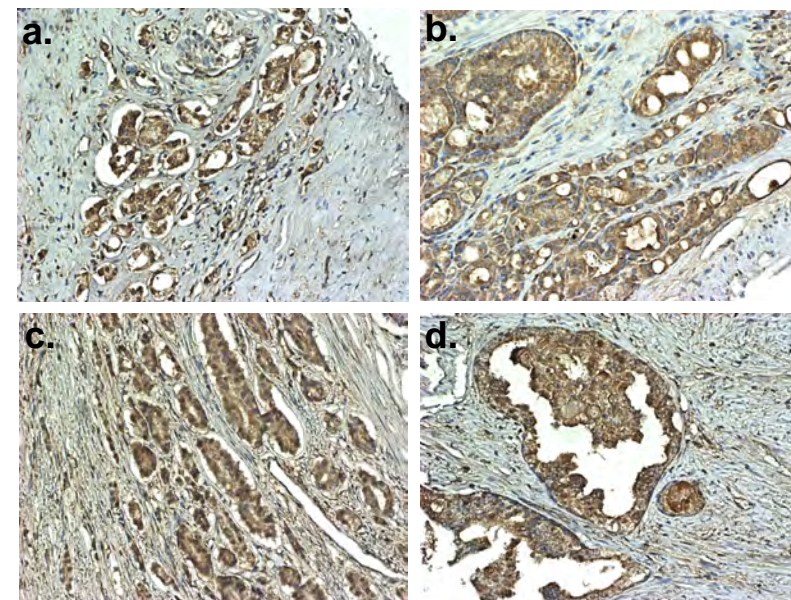
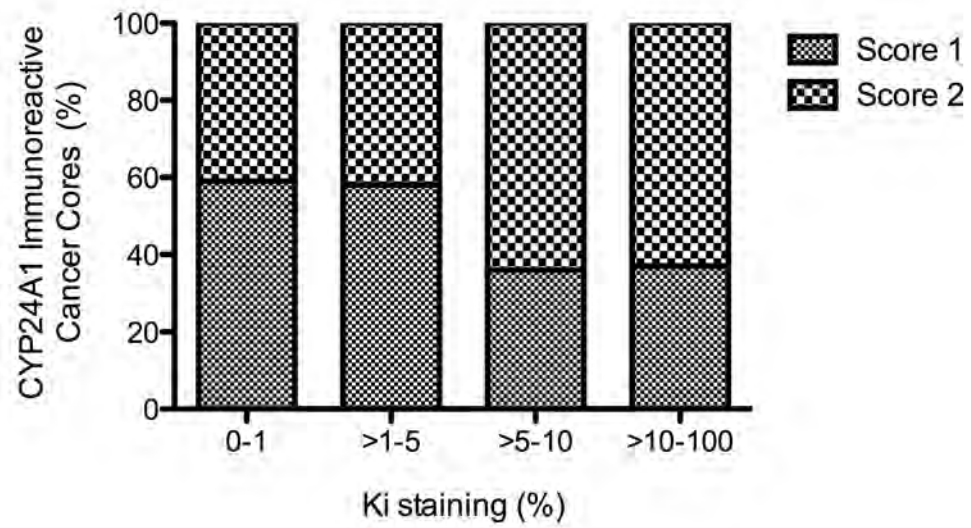


Figure 2

A.



B.

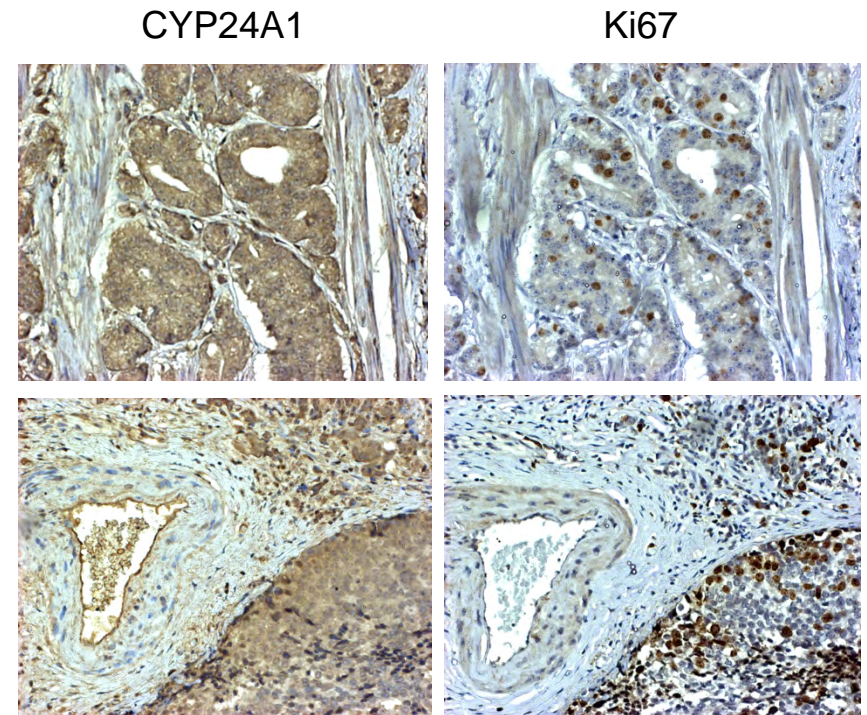
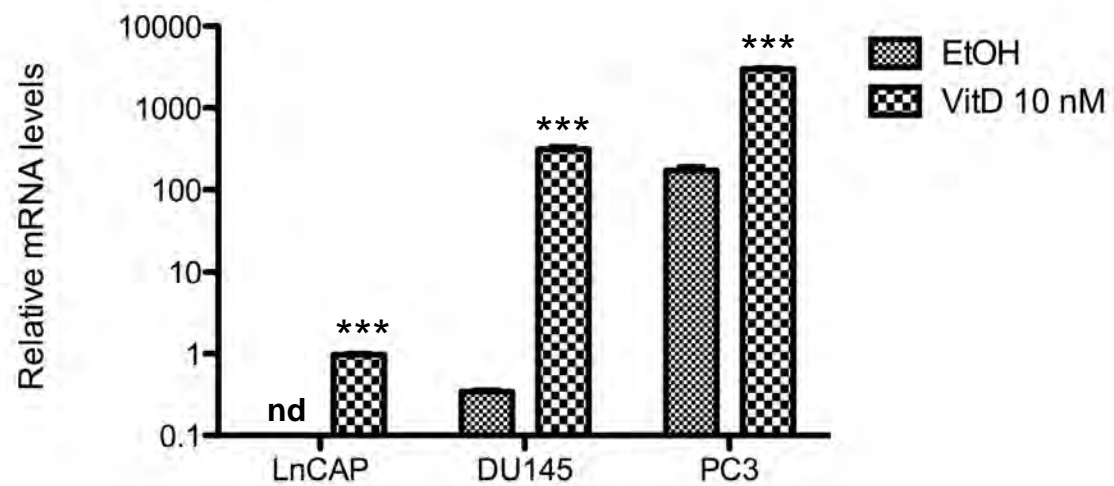
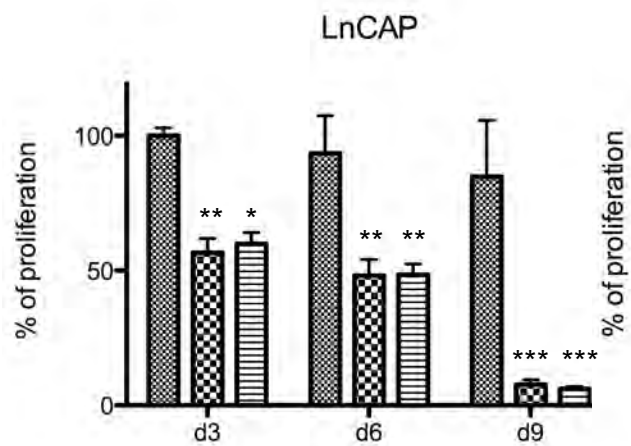


Figure 3

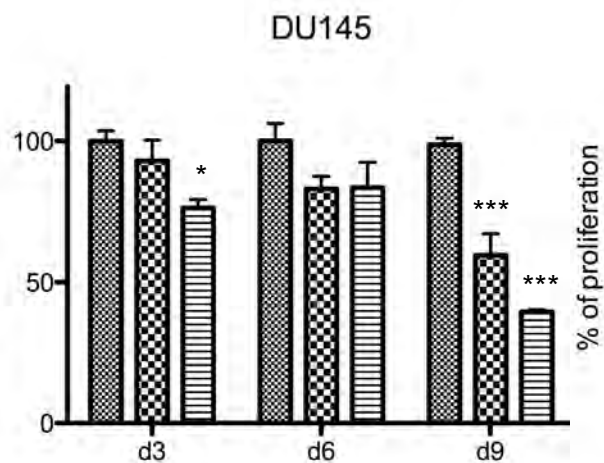
A.



B.



C.



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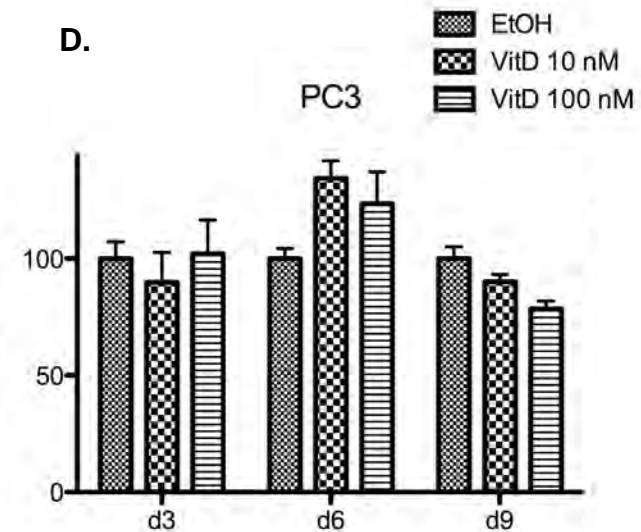


Figure 4

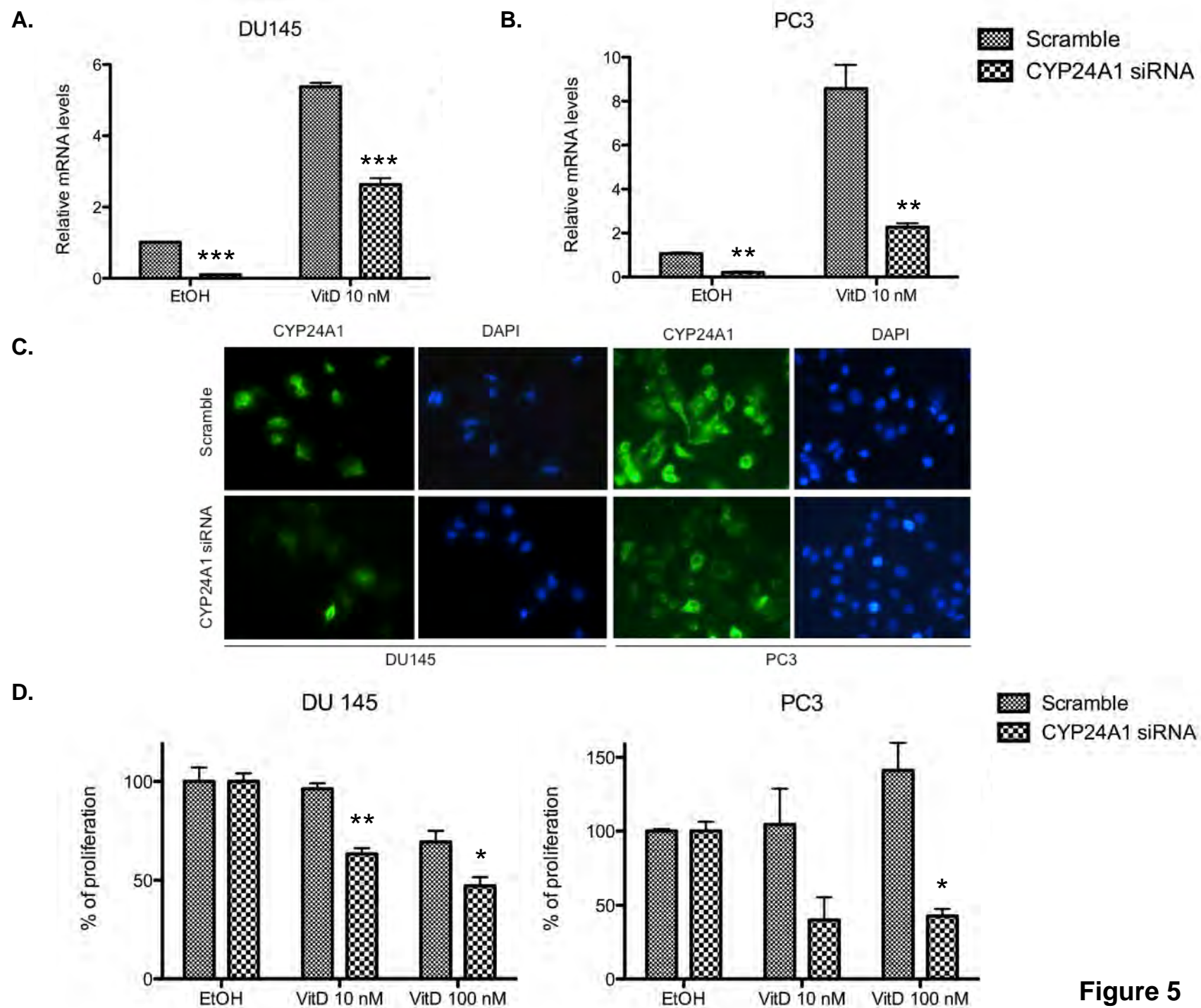


Figure 5

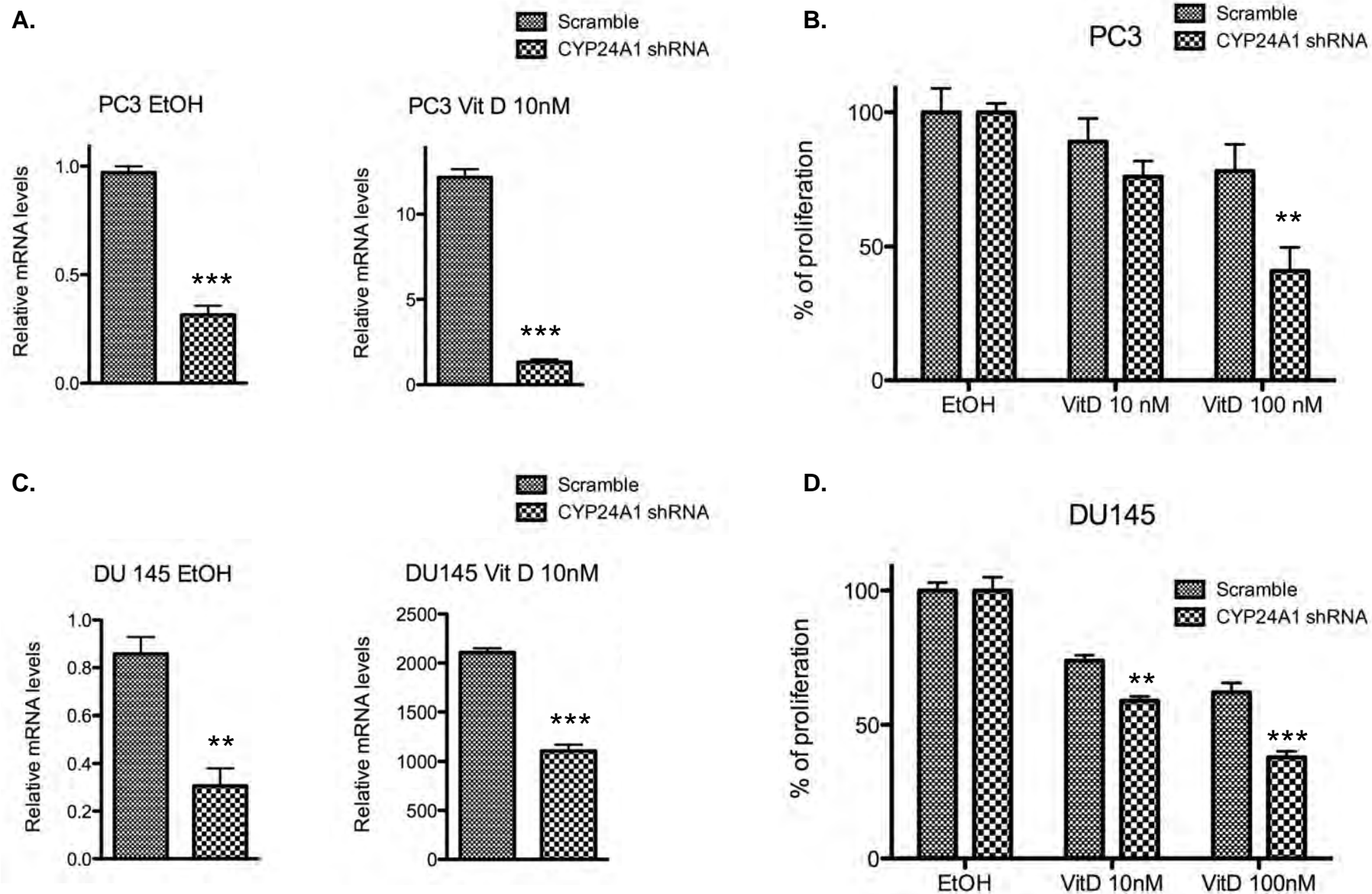


Figure 6